University of Alberta

The use of goldfish (*Carassius auratus* L.) as biosentinels for xenobiotic exposure in treated municipal wastewater and reuse water.

by

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ABSTRACT

Municipal wastewater is contaminated with many xenobiotics that can affect the physiology of living organisms. I found that goldfish (*Carassius auratus* L.) can be used as biosentinels for assessment of xenobiotic exposure in municipal final effluent (FE wastewater) and reuse water produced through membrane ultrafiltration (UF) alone (MF wastewater) or membrane UF followed by granular activated carbon filtration (MCF wastewater). Membrane UF alone was not sufficient to remove many of these chemicals from FE wastewater while activated carbon filtration efficiently reduces most xenobiotics. FE and MF wastewater contained both aryl hydrocarbon receptor agonists (cytochrome P4501A induction) and endocrine disrupting compounds (vitellogenin induction). mRNA expression of select immune genes was up-regulated in FE and MF exposed fish at early time points, while all chronically exposed fish had reduced leukocyte proliferative abilities. The infection of goldfish with *Trypanosoma danilewskyi* was found to induce both immunosuppression and immune enhancement indicating that exposure to reuse water affected the ability of fish to respond to the parasite.

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LIST OF ABBREVIATIONS

2,4-D – 2,4-dichlorophenoxyacetic acid

ANOVA – analysis of variance

APE – alkylphenol polyethoxylate

ArH – aryl hydrocarbon receptor

BaP – benzo[*a*]pyrene

BrdU – 5-bromo-2-deoxyuridine

BS – biological sciences

ConA – concanavalvin A

COX – cycloxygenase

CSF-1R – macrophage colony stimulating factor-1 receptor

CYP1A – cytochrome P450 1A

DbA – dibenz[*a,h*]anthracene

DNA – deoxyribonucleic acid

DTT – dithiothreitol

E2 – 17β-estradiol

EE2 – 17α-ethinylestradiol

EDC – endocrine disrupting compound

EDTA – (Ethylenedinitrilo)-tetraacetic acid, disodium salt dihydrate

EEQ – estradiol equivalent

ELISA – enzyme linked immunosorbent assay

ER – estrogen receptor

ERBA – estrogen receptor binding assay

EROD – 7-ethoxyresorufin-O-deethylase

E-Screen – estrogen screen

FE – final sewage effluent

GAC – granular activated carbon

GSI – gonadosomatic index

HPLC – high performance liquid chromatography

HSI – hepatosomatic index

hsp70 – heat shock protein 70

IgM – immunoglobulin M

IL-1β – interleukin-1 beta

IL-10 – interleukin-10

LPS – lipopolysaccharide

MCF – UF membrane and GAC filtered effluent

MCPP – 2-(2-methyl-4-chlorophenoxy) propionic acid

M-CSF/CSF-1 – macrophage colony stimulating factor-1

MDL – method detection limit

MF – UF membrane effluent

NADPH – nicotinamide adenine dinucleotide phosphate

iNOS – inducible nitric oxide synthase

NP – nonylphenol

NSAID – non-steroidal anti-inflammatory drug

PAH – polyaromatic hydrocarbon

PAMP – pathogen-associated molecular pattern

PBL – peripheral blood leukocyte

PCB – polychlorinated biphenyl

PCP – pentachlorophenol

PMA – phorbol 12-myristate 13-acetate

PMSF – phenylmethyl-sulfonyl fluoride

PPCP – pharmaceuticals and personal care products

RIA – radioimmunoassay

RNA – ribonucleic acid (m – messenger)

RT-PCR – reverse transcriptase-polymerase chain reaction

StAR – steroidogenic acute regulatory protein

TGFβ – transforming growth factor beta

TIU – trypsin inhibitory units

TLR-22 – toll-like receptor 22

TNFα – tumour necrosis factor alpha

UF – ultrafiltration

Vtg – vitellogenin

WWTP – wastewater treatment plant

YES – yeast estrogen screen

CHAPTER ONE

GENERAL INTRODUCTION AND OBJECTIVES

1.1 Introduction

The study of toxicology, or the effects of various harmful chemicals on living organisms, is one of the most widely studied aspects in environmental sciences. In the past, many toxicological studies examined the adverse effects of pollutants that were derived from industrial or chemical manufacturing processes such as pulp and paper mill effluent or heavy metals. Recently, however, the presence of endocrine disrupting compounds as well as pharmaceuticals and personal care products in the environment has generated a great deal of interest. Many studies have demonstrated that not only are xenobiotics (man-made chemicals) present in municipal wastewater and the aquatic environment (1-3), but that these chemicals can have significant and long-lasting impacts upon aquatic organisms such as freshwater fish (4-8).

Many parts of the world make use of reuse, or recycled, municipal wastewater for non-potable purposes such as landscape and food-crop irrigation, toilet flushing, and industrial cooling processes (9). Other parts of the world use recycled water for groundwater recharge in arid regions (10) or even utilize it directly after treatment for potable purposes (11). Indirect potable water reuse may also occur when cities withdraw their drinking water from rivers that are contaminated with xenobiotics from upstream municipalities discharging sewage effluent into the water system. Additionally, some xenobiotics have retention times of greater than six years within groundwater, and may enter the water table through soil-aquifer treatment (10) or groundwater aquifers through bank filtration (12). These chemicals have even been detected in trace amounts in treated drinking water (12; 13). In urban areas that experience low surface water flow rates and high sewage effluent outputs, such as Berlin, Germany, not only is there a greater risk to exposed aquatic animals because of a reduced dilution effect, but there is also a greater risk of xenobiotic contamination of nearby potable water supplies (2).

As mentioned, recycled wastewater contains numerous xenobiotics at concentrations that have the potential to induce physiological effects in exposed

organisms (14-16). Xenobiotic exposure may be associated with physiological changes in animals, specifically teleost fish, as reviewed by (4; 1; 5-7; 17; 18). These chemicals can affect such diverse aspects of physiology as tissue integrity (19; 20), metabolism and xenobiotic clearance (5; 21; 22), reproduction (23-26), and the immune response (27-31).

Aquatic organisms such as goldfish may be used as biosentinels for potential adverse effects of wastewater and recycled water exposure, and as such, act as a toxicological 'canary in a coalmine'. Previous studies using aquatic organisms have been done in static systems (32). Use of a real-time flow-through setting, done in my study, allows for more realistic xenobiotic exposure protocols. This is because fish are exposed to xenobiotics at full strengths with minimal chemical degradation that may otherwise occur due to photodegradation or extended bacterial enzymatic activity. In a flow-through exposure system, fish are also more likely to be exposed to ambient fluxes of xenobiotics that occur in the operating wastewater treatment plant.

If continued use of recycled water occurs and chemicals in the effluent are not properly eliminated, there is a potential risk of xenobiotic accumulation in the drinking water supply. Consequently, methods for reducing the risks of xenobiotics in the natural environment and society are of utmost importance. Through use of both chemical analysis and a number of other animal models including goldfish, society may be able to reduce the impact of industrialization on environmental ecosystems as well as ensuring the safety of our drinking water supply for generations to come.

1.2 Objectives of the Thesis

The main objective of my thesis work was to examine the physiological effects of exposing goldfish, *Carassius auratus* L., to treated municipal sewage final effluent for various periods of time at a pilot scale. My second objective was to examine the ability of reuse wastewater, as produced through membrane ultrafiltration (UF) or membrane UF followed by granular activated carbon (GAC) filtration, to reduce or eliminate the observed physiological effects elicited by exposure to final effluent wastewater in goldfish. The specific aims of my thesis were:

1) To examine the effect of exposure of goldfish to final effluent or wastewater produced by various filtration techniques over a period of 90 days on P450

(CYP1A) enzymatic activity in liver microsomes, as measured using the 7-ethoxyresorufin-O-deethylase (EROD) assay, a common biomarker of aryl hydrocarbon exposure.

2) To examine induction of the estrogenic contamination biomarker vitellogenin in male goldfish exposed to various wastewater treatments for up to 90 days.
3) To examine the mitogen-stimulated proliferative ability of peripheral blood leukocytes, isolated from goldfish exposed to final effluent or reuse wastewater for up to 90 days.
4) To investigate the expression of key immunological genes following exposure to various wastewater treatments for up to 90 days using semi-quantitative RT-PCR techniques. A variety of genes were examined including toll-like receptor-22 (TLR-22), tumor-necrosis factor alpha (TNF-α), colony stimulating factor-1 receptor (CSF-1R) and granulin.
5) To determine the effects of final effluent or reuse water exposure on the immunocompetence of goldfish following challenge with the protozoan fish parasite *Trypanosoma danilewskyi*.

1.3 Outline of Thesis

The second chapter of the thesis is a literature review and includes a general description of xenobiotics found in both municipal sewage treatment plant wastewater and the aquatic environment, common toxicological assays used to detect or to determine the *in vitro* and *in vivo* physiological effects of xenobiotics, and a review of the effects of xenobiotics on teleost fish. In chapter three, I outline the experimental set up at Gold Bar Waste Treatment Plant and describe the various materials and methods used to determine the effects of wastewater exposure on goldfish. The fourth chapter consists of a chemical analysis of the xenobiotics to which the fish were exposed to in the various wastewater treatments at each of the sampling time points that occurred during this study. In chapter five, I describe the results of a two common toxicological assays used to examine the effect of exposing goldfish to different wastewater treatments, namely P450 (CYP1A) induction (EROD assay) and vitellogenin induction in male fish. Chapter six contains the results of the peripheral blood leukocyte proliferation assay and the semi-quantitative

gene expression of the immunological genes TLR-22, TNF-α, CSF-1R and granulin in the kidney of goldfish. Chapter seven describes the parasite infection studies (*Trypanosoma danilewskyi*) and chapter eight is a general discussion of the results, the importance of the use of biosentinels for aquatic toxicology and reuse water, and future directions for this study.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Introduction*

An increase in global temperature due to warming trends may be associated with a loss or reduction in fresh water resources. Furthermore, irrigation of food crops in progressively arid regions, rapid population growth within urban centers, and the use of fresh water for industrial purposes will result in access to clean, potable water becoming an increasingly important issue in the future. For example, Chen *et al.* (33) ran predictive models regarding the availability of fresh water and municipal water use in a major Canadian city, Calgary, Alberta. Over the next sixty years the municipal population is expected to double, yet in order to maintain sustainable growth the authors predicted that the city will have to reduce their per capital water use to less than half of its current level.

Due to the increasing scarcity of adequate freshwater supplies, reuse of municipal treatment plant wastewater is becoming an increasingly common practice in many parts of the world. Reclaimed water is used for such non-potable purposes as agricultural or landscape irrigation, industrial activities, groundwater recharge, recreational and environmental use (man-made lakes, etc), in addition to potable reuse purposes (34). Some of the regions of the world that currently utilize reuse water include the United States (35; 36), Mexico (37), Italy (38), China (39), Taiwan (40), Japan (9), and southern Africa (11; 41).

Municipal wastewater can contain thousands of different xenobiotics, including pharmaceuticals and personal care products (PPCP), endocrine disrupting compounds (EDCs), heavy metals, and pesticides (14; 1; 15; 42; 2; 16; 3). The concentrations of these compounds can range from trace levels (pg/L) to micrograms per liter. Reuse water may also contain xenobiotics and these compounds can subsequently enter the environment through reuse water practices. For example, wastewater-derived pharmaceuticals have been detected in both the soil irrigated with reuse water (35) and in agricultural runoff following precipitation events (43). Several pharmaceuticals have a high degree of environmental persistence and may even be detected in groundwater supplies (12; 10) or at trace levels in treated drinking water (12; 13).

Many studies have demonstrated the potential for these compounds to affect animal health, as reviewed in (4-8), at environmentally relevant concentrations (44-47). Consequently, if the reuse of water increases and xenobiotics are not properly removed during drinking water or sewage treatment, these compounds could potentially accumulate in the environment or drinking water supply, and have unknown effects on exposed aquatic organisms or humans. As such, it is of paramount importance that the safety of the drinking water supply and the environment is assured.

2.2 *Pollutants in Wastewater*

Municipal wastewater is known to be contaminated with numerous pollutants that may include pesticides (48), pharmaceutical residues (49; 50), and heavy metals (51). These human-manufactured chemicals, or xenobiotics, can potentially enter the environment through a number of different routes including municipal sewage treatment plant effluents (14; 15; 10; 16; 52; 49), leachate from landfills (53), runoff from agricultural land fertilized with solid waste or irrigated with reuse water (43), human medicines, and veterinary medications used in agriculture (54). Many large-scale agricultural practices such as concentrated animal feeding operations also have the potential to contaminate ground or river water sources (55) and to affect the physiology of animals exposed in the downstream environment (56). Many pharmaceutical drugs form conjugates with polar molecules in the human body and are eliminated in the urine. During sewage treatment these pharmaceuticals may be cleaved from the polar molecules (57) and eliminated to varying degrees through biodegradation or adsorption to particulate matter prior to discharge into the environment (2).

Hundreds of thousands of xenobiotics may be found at trace levels in municipal wastewater or the environment; however, many of these chemicals are below detection limits or detection is limited by analytical methodology. While the concentration of individual xenobiotics in municipal sewage effluent may be low, the sheer quantity of effluent released into the watershed on a daily basis can result in considerable quantities of these bioactive compounds entering the environment. In fact, river systems are often contaminated with xenobiotics through the discharge of treated sewage effluent (14; 58; 15; 44; 49; 59; 13; 50; 60) and these xenobiotics may be found at or approaching levels

that exert biological effects in *in vitro* studies. For the purpose of this review, xenobiotics in municipal wastewater can be roughly classified as pharmaceuticals and personal care products (PPCP), endocrine disrupting compounds (EDCs), pesticides, and heavy metals and organic pollutants.

2.2.1 Pharmaceuticals and personal care products

Pharmaceuticals and personal care products are perhaps the most diverse major class of xenobiotics in the environment, and may be found at relatively high levels in municipal wastewater. PPCPs can be further subdivided into a number of classes, including non-steroidal anti-inflammatory drugs (NSAIDs), anti-epileptics, and lipid regulating drugs.

2.2.1.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are analgesic and antipyretic anti-inflammatory agents that selectively or non-selectively inhibit cyclooxygenase enzymes. Cyclooxygenase (COX) enzymes naturally function to convert arachidonic acid into the unstable intermediate prostaglandin G_2 (61) and have been found to exist in at least two subtypes in mammals and lower vertebrates such as fish: the constitutively expressed COX-1 enzyme and the inducible COX-2 enzyme. Research has shown that COX-1 aids in the production of prostaglandins that are involved in a large number of physiological processes including reducing gastric acid secretion, increasing gastric mucus production, and influencing renal blood flow (among many others), whereas COX-2 is primarily induced in activated macrophages and inflamed tissues in response to proinflammatory cytokines. However, there is strong evidence that COX-1 may be involved in inflammation and COX-2 may be constitutively expressed in many tissues in the body, as reviewed by (62; 63). Only a select number of these pharmaceuticals are selective COX-2 inhibitors (celecoxib/Celebrex®, valdecoxib/Bextra® and the recently withdrawn rofecoxib/Vioxx®); the majority of NSAIDs are non-selective COX inhibitors.

Diclofenac

Diclofenac is a non-selective COX inhibitor commonly prescribed in the treatment of arthritis and general pain management, as well as in veterinary medicine. It

has been found in municipal wastewater in concentrations ranging from 0.12 µg/L to 1.5 µg/L (14; 64; 65; 49; 59; 13; 60). In the environment, diclofenac has been found with maximal concentrations ranging from 0.194 µg/L to 1.20 µg/L (14; 66). In a recent province-wide survey of municipal wastewater treatment plants (WWTPs) and receiving waters across Alberta, Canada, diclofenac was detected in effluent at a maximum concentration of 0.429 µg/L (Fish Creek WWTP, Calgary) and as high as 0.090 µg/L in river systems downstream of effluent discharge sites (downstream of Edmonton; (59)). Removal efficiency of diclofenac is generally low in sewage treatment plants, with removal rates of less than 20% of influent concentrations (49; 67).

Naproxen

Naproxen is a pharmaceutical used in the relief of pain, fever and inflammation, such as caused by rheumatoid arthritis, injury, and menstrual cramps. This NSAID may be obtained through a doctor prescription or sold as an over-the-counter drug (Aleve®). Naproxen is commonly found in municipal wastewater in Europe (14; 49; 13), Canada (16; 59; 60) and America (10) with concentrations ranging from 0.25 µg/L (49) to maximum concentrations of 33.9 µg/L (16). Even though sewage treatment results in high (93%) removal efficiency of naproxen compared to influent concentrations (49), this drug has been found in surface waters near Canadian sewage treatment plants, with median concentrations as high as 0.207 µg/L, and maximum concentrations of 0.551 µg/L (66). In German rivers and streams (not necessarily closely located to sewage treatment plants) median and maximum naproxen concentrations of 0.070 µg/L and 0.39 µg/L, respectively, hjave been reported (14). In a survey of provincial waters done by the Government of Alberta (59) maximal levels of naproxen were as high as 2.67 µg/L in a Calgary WWTP and as high as 0.11 µg/L in river water downstream of the city of Edmonton

Ibuprofen

Ibuprofen, a common over-the-counter medicine for pain relief sold under such brands as Advil® and Motrin®, has been found on numerous occasions in sewage treatment plant effluents (14; 10; 16; 65; 49; 59; 60) as well as the environment (14; 68; 66; 59; 13). Ibuprofen has a very high removal efficiency (>90%) from sewage treatment

plant influents (69; 49), but is commonly used as an analgesic and consequently exists in substantial concentrations in sewage effluent, ranging up to maximal levels of 24.6 μg/L [median concentration 4.0 μg/L; (16)]. Environmental concentrations of ibuprofen have been detected in surface waters receiving both treated and untreated sewage with concentrations reaching up to 0.790 μg/L [median concentration 0.141 μg/L; (66)], and even at trace levels in agricultural runoff from fields treated with treated wastewater (43). A previous survey of WWTP effluent from Edmonton, Alberta, revealed ibuprofen concentrations as high as 1.76 μg/L in the final effluent and 0.269 μg/L in waters downstream of the city (59).

Other analgesics/antipyretics

Severasl other anti-inflammatory drugs have been detected in sewage treatment plant effluents and the aquatic environment. For example, Oros *et al.* (70) detected acetaminophen (Tylenol®) at a mean concentration of 0.172 μg/L in the San Francisco Bay estuary. Others have detected compounds such as acetylsalicylic acid in effluents from German sewage effluents [median concentration 0.22 μg/L, maximum concentration 1.5 μg/L; (14)]. Salicylic acid, the de-acetylated form of acetylsalicylic acid, has also been detected in sewage treatment plant effluents (14; 16), with maximal concentrations of 59.6 μg/L [median concentration 3.6 μg/L; (16)], as well as in German rivers and streams [median concentration 0.025 μg/L, maximum concentration 4.1 μg/L; (14)]. It should be noted that salicylic acid is also found in skin and hair-care products and is a natural compound in plants. Consequently, the origin of this compound in wastewater or in the environment cannot be solely attributed to pharmaceutical usage.

2.2.1.2 Anti-epileptic drugs

One of the most commonly detected xenobiotics in municipal wastewater and the environment is the anti-epileptic/anti-mania drug carbamazepine (14; 68; 16; 66; 65; 52; 49; 43; 59; 50; 60). Carbamazepine acts as a selective serotonin re-uptake inhibitor to reduce incidences of seizures as well as acting as a mood-stabilizer. In sewage treatment plant effluents, concentrations of this compound have been found as high as 6.3 μg/L [median concentration 2.1 μg/L (14); or, in Alberta, 3.29 μg/L in effluent from the Red Deer WWTP (59)]. Environmental concentrations are, in general, ten fold lower than

those found in sewage effluents, but concentrations as high as 1.1 µg/L (median 0.25 µg/L) have been found in German rivers and streams (14). In southern Alberta rivers, levels as high as 0.206 µg/L have been reported (59). Removal efficiency of carbamazepine in sewage waste treatment plants is low, generally less than 15% (69), and likely contributes to its prevalence in wastewater samples and the environment.

2.2.1.3 Lipid regulating drugs

Gemfibrozil and bezafibrate are lipid-lowering drugs commonly detected in wastewater samples. In humans, these drugs act to lower the levels of low-density lipoprotein, cholesterol and fatty acids in the blood. Gemfibrozil has been found in numerous sewage treatment plant effluents (14; 16; 49; 59; 60), with maximal concentrations detected reaching as high as 1.5 µg/L [median concentration 0.40 µg/L (14); or 0.813 µg/L in Alberta (Red Deer WWTP; (59)]. Environmental concentrations of this chemical have ranged as high as 0.112 µg/L to 0.51 µg/L respectively, (14; 66). In Alberta watersheds, however, gemfibrozil has only been found at levels as high as 0.067 µg/L [upstream of Medicine Hat, AB; (59)].

Sewage wastewater effluent concentration of bezafibrate have reached as high as 4.6 µg/L [median concentration 2.2 µg/L; (14)] with lower concentrations found in many other surveys of municipal effluents in Europe (65; 50) and Canada (16; 59; 60). This compound has also been detected in final effluent from Gold Bar WWTP; the concentration of bezafibrate found in a Government of Alberta survey in 2002 was found to be 0.547 µg/L; Edmonton, Alberta (59). Regardless of its fairly high removal efficiency (~90%) in sewage treatment plants (65), bezafibrate is commonly found in aquatic ecosystems (14; 68; 66; 59; 13). Furthermore, environmental concentrations of this drug can be substantial; Ternes (14) detected levels of bezafibrate up to 3.1 µg/L (median concentration 0.35 µg/L) in rivers and streams of central Germany, though an Alberta river survey only yielded concentrations as high as 0.029 µg/L [downstream of Edmonton, AB; (59)].

2.2.2 Endocrine disrupting compounds

Endocrine disrupting compounds (EDCs) are defined as substances "having the ability to disrupt the synthesis, secretion, transport, binding, action or elimination of

natural hormones in an organism, or its progeny, that are responsible for the maintenance of homeostasis, reproduction, development or behaviour of the organism" (71). Estrogenic compounds are a specific class of EDCs, as they specifically interfere with estrogen homeostasis or the reproductive physiology of fish resulting in preferential feminization of exposed organisms. They may function as estrogen mimics, estrogen receptor agonists or testosterone/testosterone receptor antagonists. In general, organisms are very sensitive to estrogenic compounds; physiological effects may be observed at very low concentrations (down to the nanamolar range). In addition to natural estrogens such as 17β-estradiol (E2), estrone and estriol, a large number of synthetic xenobiotics can also function as estrogen mimics or may interact with other pathways that impact estrogen and its physiological effects. These xenobiotics include the contraceptive hormone 17α-ethinylestradiol (EE2), surfactants such as alkylphenol polyethoxylates (APEs) and nonylphenols (NP), polychlorinated biphenyls (PCBs), phthalic esters, dioxins, and naturally occurring compounds in plants (72; 73).

In municipal wastewater, natural estrogenic compounds and contraceptive hormones are of primary concern due to their excretion from the human population. Estrogenic compounds have been detected in sewage treatment plant effluents from many countries, including Europe, Canada, Brazil and Australia (74; 15; 42; 48; 59; 75). In general, the mean concentration for EE2 was below 2 ng/L, but was detected as high as 64 ng/L in sewage treatment plant effluent (15). Estrogens such as estradiol can be released from conjugates during the sewage treatment process, resulting in the release of the active parent compound and increasing estrogen concentrations with further wastewater treatment (57). Environmental concentrations of EE2 are generally much lower than those detected in the sewage treatment plant effluents; however, in times of low river or stream flow rates, municipal wastewater effluent may make up a significant proportion of the total flow and, as such, may significantly impact living organisms. Environmental concentrations of E2 usually range between below method detection limits to 2.2 ng/L (42); however, concentrations of E2 as high as 3 ng/L in agricultural runoff from fields irrigated with municipal wastewater have been reported (43).

Androgenic compounds, though less studied than estrogenic compounds, have also been detected in municipal sewage treatment plant effluent (76-80). Concentrations

of androgenic compounds are measured in dihydrogentestosterone equivalents, and studies have detected these compounds ranging between below detection limits to ~3 ng/L in treated final wastewater effluent, and as high as 160 ng/L in effluent that did not receive secondary treatment (80). Removal efficiency for these androgenic compounds was high (96-99%) (80).

2.2.3 Pesticides

Pesticides such as insecticides, fungicides and herbicides are the only known toxic compounds that are purposefully introduced into the environment. These chemicals may be applied in both agricultural and municipal settings for use in insect or weed control, and have been shown to be present in detectable levels in the aquatic environment (76-78). For example, the pesticide carbofuran has been transiently detected at levels up to 26 μg/L in agricultural stream runoff following heavy rain (78). Unlike many pharmaceutical products that are discharged into the environment at select point sources such as wastewater treatment plants, pesticide usage also occurs in agricultural settings resulting in these chemicals potentially being detected in environments that are substantial distances from sewage treatment plants or urban centers.

2.2.4 Heavy metals and organic pollutants

An extensive body of research has examined heavy metal contamination in the environment. These metals, which may include mercury, lead, aluminum, cadmium, copper, zinc and nickel, among many others (81-86; 39), have been linked to adverse health effects in both animals and humans (87). These metals have also been detected in municipal effluents. For example, Gagné *et al.* (51) found that treated effluent from the city of Montreal and surrounding municipalities contained substantially higher levels of the heavy metals cadmium (1 μg/L), chromium (3 μg/L), copper (23 μg/L), and zinc (32 μg/L) than upstream water samples. Furthermore, these authors observed that these chemicals bioaccumulate in freshwater mussels (*Elliptio complanata* and *Dreissena polymorpha*) exposed 5 km downstream of the discharge site.

In addition to the previously described chemicals, municipal wastewater effluent may also contain polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) that have the potential to harm aquatic organisms (88). Though their use has

been largely restricted for the past thirty years, PCBs are commonly used as coolants in electrical transformers and capacitors and are classified as persistent organic pollutants. PAHs, another class of persistent organic pollutant, may be found in crude oils or formed through the incomplete combustion of petroleum, coal, wood, or other carbon-based fuels. PCBs and PAHs have been detected in treated municipal effluent and receiving surface waters (89) and in untreated sewage effluent (90). In a study of treated sewage effluent from the city of Montreal, mean concentrations of 13 PCBs and 14 PAHs were found to be 1.34 ng/L and 326 ng/L, respectively, whereas these chemicals were significantly reduced in the St. Lawrence River and completely diluted 11 km downstream of the effluent outfall site (89).

However, it is important to note that many organic pollutants such as PCBs and chlorinated pesticides are lipophilic in nature and thus have low water solubility; as such, these chemicals tend to have a high affinity for organic matter (91). Because of this high affinity for organic matter, these compounds tend to not concentrate in liquid effluent, and instead are often detected in treated municipal sewage sludge (91; 92).

2.3 *Use of Different Assays for the Assessment of Pollutants*

Xenobiotic contamination in wastewater effluents or the environment can be through chemical methods, for example, using gas chromatography-mass spectrometry. However, the detection of xenobiotics in a water sample does not necessarily correlate with a biological effect in an organism exposed to that water sample. In order to properly assess the biological impacts of xenobiotic exposure, many *in vitro* and *in vivo* biomarkers have been developed. Biomarkers are described as "xenobiotically induced variations in cellular or biochemical components or process, structures, or functions that are measurable in a biological system or sample" (93). An essential assumption regarding the use of biomarkers is that they respond in a dose-dependant manner to xenobiotic concentration gradients (94). The theory of hormesis, however, suggests that biological effects may differ substantially depending upon the concentration of chemical used (206). For example, compounds that may result in harmful effects on biological systems when applied in high doses may have beneficial effects when applied in low

concentrations. Consequently, care must be taken when predicting the biological effects of a chemical when a full dose-response study has not been conducted.

In complex mixtures of xenobiotics, such as those found in municipal sewage treatment plant effluent or bodies of water that receive sewage effluent, xenobiotics may act in an additive, inhibitory or synergistic manner (95; 96). Although chemical analysis may indicate that all chemicals are below their "no observable effects concentrations", these chemicals may still impact living organisms through additive or synergistic effects.

Many studies have shown both acute and chronic impacts of aquatic pollutants on living organisms such as fish, as reviewed by (1; 5-7; 3; 8). Pharmaceutical and pesticide chemicals pose a special risk to organisms, as these chemicals may impact the physiology of organisms at very low concentrations and also have longevity in animals and often the environment. Pharmaceutical compounds may also cause unknown physiological effects in non-target aquatic organisms (1; 8) or break down into more toxic compounds in the environment (97). Physiological effects of xenobiotics may be assessed using biomarkers and a variety of methods and assays, roughly grouped into either *in vitro* or *in vivo* assays, each with advantages and disadvantages.

2.3.1 *In vitro* assays

A number of toxicological assays make use of *in vitro* methodology including yeast or tissue derived cell lines such as the commonly used estrogen-screen (E-Screen) and yeast estrogen screen (YES) assays for estrogens. In general, *in vitro* assays are simple, cheap to maintain and usually have a low intra/inter-assay variation due to the use of homogeneous cell cultures. Consequently, *in vitro* assays may be used to efficiently screen large numbers of test samples for xenobiotic contamination. Additionally, these assays are usually sensitive and responsive to low levels of chemicals. Consequently, *in vitro* assays can be used if compounds of interest are present below their method detection limits (MDLs) but additive or synergistic effects are predicted to occur.

However, *in vitro* methods are limited in that results obtained may not necessarily reflect how whole organisms will respond to similar doses of xenobiotics. Animals may metabolize or excrete compounds, reducing the chemicals' effects on the animal's physiology. It is difficult to predict non-specific effects of xenobiotic exposure that may occur in whole organisms. Furthermore, when cell lines are cultured for extended

periods of time, genetic drift may occur and result in the culture no longer responding in a similar or predictable manner as earlier cultures. Since *in vitro* assays only work with single cell types, intricate interactions between different cell types commonly found in tissues are absent. Consequently, *in vitro* systems cannot easily be used to test physiological responses that require complex cellular interactions. Nevertheless, a number of *in vitro* assays have been developed to examine the effects of xenobiotics on living cells.

2.3.1.1 Comet assay for genotoxicity

The comet assay makes use of either cell cultures or *ex vivo* cells (tissue cells isolated from organisms and subsequently cultured *in vitro*) in order to deduce the genotoxicity of specific chemicals. It detects damage to a cell's deoxyribonucleic acid (DNA) by measuring the migration of nuclear DNA out of the nucleus. In short, cells are imbedded in agarose on microscope slides, exposed to xenobiotics or other potentially damaging substances, and then made permeable with detergents. The cells are lysed and subjected to electrophoresis resulting in the smaller, damaged DNA fragments traveling out of the nucleus and creating a visible streak similar to that of a comet's tail (98). This assay can be used to detect DNA damage resulting from direct exposure of cells to xenobiotics *in vitro* or damage in cells isolated from organisms exposed to xenobiotics *in vivo*. For example, a number of xenobiotics such as the PAH benzo[a]pyrene, as well as non-extracted and non-concentrated polluted river water samples have been found to induce genotoxicity in the rainbow trout gonad cell line-2 (99).

2.3.1.2 Endocrine disrupting compound (EDC) assays

Interest in EDCs has greatly increased within the last decade, as compounds such as estrogens have been detected numerous times in the environment and/or municipal wastewater (74; 64; 42; 48). Not surprisingly, a large number of *in vitro* assays have been developed to detect these compounds in water samples.

Competitive estrogen receptor binding assay (ERBA)

As the name suggests, the competitive estrogen receptor binding assay (ERBA) measures the binding of compounds suspected of being estrogen receptor agonists or antagonists to a specific cellular receptor, such as sheep uterus estrogen receptors. The

degree of binding of the xenobiotic to the receptor is then compared to that of 17β-estradiol, thereby allowing an estradiol equivalent (EEQ) value to be assigned to the compound in question. A drawback of the ERBA is that this assay cannot differentiate between an agonist and an antagonist, as both substances have high affinity for the estrogen receptor. In order to determine antagonistic or agonistic effects of chemicals on receptors, assays involving whole cells must be used.

Estrogen-screen (E-Screen) assay

The estrogen screen, or E-screen assay, is a sensitive and stable method used to measure the degree of estrogenicity of a compound or sample by using an estrogen sensitive cell line that proliferates in the presence of estrogenic compounds. One such example of a cell line is the human breast cancer cell line MCF-7. Significant increases in cell proliferation have been detected with concentrations of 17β-estradiol as low as 1 pM [~0.07 EEQ or 0.27 pg/ml; (100)]. Additive behaviour of multiple xenobiotics in mixtures has also been detected using the E-screen (101; 100).

Yeast estrogen screen (YES) assay

In the YES assay, cells are transfected to produce a readily detectable protein when a test compound activates a known receptor. Yeast (*Saccharomyces cerevisiae*) cells are usually transfected with an expression vector coding for the human estrogen receptor as well as with an estrogen-sensitive promoter that is linked to the gene for β-galactosidase or another appropriate enzyme. Activation of the estrogen receptor by natural or synthetic estrogenic compounds therefore leads to an increase in β-galactosidase (or other enzyme) gene expression, and a corresponding quantitative increase in catalytic activity when the yeast cells are exposed to the appropriate substrate (102).

2.3.2 *In vivo* assays

In vivo toxicology assays involve the exposure of living organisms to different treatments such as increasing xenobiotic concentration or to complex chemical mixtures for various periods of time. These types of assays are commonly used for monitoring of xenobiotics in wastewater or the environment, and to determine the effects of specific

pollutants on the physiology of living organisms. *In vivo* assays allow the measurement of both acute and cumulative effects of a chemical or mixture on complex physiological processes within the plant or animal, as well as on the overall fitness of the organism. Xenobiotic mixtures can have synergistic, additive or inhibitory effects that may not necessarily be predicted using mathematical modeling or *in vitro* methodology alone. Also, use of complex multicellular organisms such as fish allows multiple assays to be preformed using the same individuals, for example, by using different tissues for different assays. This potentially creates a wealth of complex information while reducing sample size and increasing scientific efficiency.

Disadvantages of *in vivo* methodology include intra and inter-specific variation; fish species used in toxicological experiments are outbred and this heterogeneity may be reflected in large variation in experimental results. Furthermore, variation between different species' susceptibility to pathogens and their sensitivity to xenobiotics, as well as seasonality effects can make extrapolations of the effects of chemicals on different fish species and to human populations problematic. Finally, *in vivo* assays are more costly and labour-intensive care since the experiments often involve long-term exposures to the xenobiotics.

In vivo assays commonly focus on indices of animal fitness such as survivorship, hepatosomatic and gonadosomatic indexes, reproductive development and sensory physiology such as olfactory ability. Behavioural changes like feeding, aggression and reproductive behaviour may also indicate the animals' overall fitness. Alternatively, biochemical alterations such as basal metabolic rate, relative levels of reactive oxygen intermediates in tissues, plasma hormone levels, reproductive cell viability, various indexes of immunological competence and the production of biomarkers associated with xenobiotic removal are but a few physiological processes that also may be affected by xenobiotic exposure.

2.3.2.1 7-Ethoxyresorufin-O-deethylase (EROD) activity

One of the most widely accepted methods of determining if an organism has been exposed to environmental contamination is the 7-ethoxyresorufin-O-deethylase (EROD) assay. This assay is a semi-quantitative measure of the degree of planar hydrocarbon exposure to which an animal has been subjected. In general, the EROD assay is more

sensitive than chemical analysis of contaminants in the tissues of an organism due to the fact that xenobiotics may be degraded at different rates within tissues through phase I and phase II metabolism, and as such, chemical analysis alone might underestimate the level of exposure. Furthermore, different analytical methods may be limited in the chemicals the method is capable of accurately detecting.

Specifically, the EROD assay measures the catalytic activity of the cytochrome P450 (CYP1A) enzyme family to convert a substrate (7-ethoxyresorufin) to a measurable fluorescent product (resorufin). CYP1A enzymes are members of the cytochrome P450 multigene family of enzymes and are involved in the removal of xenobiotics from an organism. The CYP1A enzymes are found in all organisms studied to date and are involved in metabolizing xenobiotics as well as endogenous molecules such as fatty acids and steroid molecules (103). In fish, P450 enzymes are found in many tissues such as the kidney, gill and gastrointestinal tract, but are found in their highest levels in liver tissue (104). CYP1A is involved in biotransforming aromatic hydrocarbons into more excretable forms, either by exposing the toxicant to a small polar group or by adding a polar group to the molecule. This polar group enhances the solubility of the xenobiotic, allowing for enhanced secretion of the substance (105). However, the addition of a polar group may result in a molecule with not only increased solubility, but also more toxicity than the parent compound (106).

CYP1A protein production is induced in response to xenobiotic exposure, specifically in response to xenobiotics with a planar hydrocarbon ring structure. EROD inducing xenobiotics include planar PCBs, PAHs, pesticides and metals, as well as natural plant compounds (5). These chemicals bind to an aryl hydrocarbon receptor (AhR) that sets off a cascade of protein reactions, eventually leading to the binding of a transcription factor to specific regions of DNA coding for a variety of proteins involved in xenobiotic removal, including CYP1A. This increased CYP1A gene expression leads to a corresponding increase in enzymatic catalytic activity, as reflected by the EROD assay (107).

A large number of studies have outlined the effects of exposing living organisms, specifically fish, to planar hydrocarbons or other xenobiotics and the resulting effects on EROD activity (5). Individual chemicals may be ranked according to the level of EROD

activity in exposed fish compared to control fish. Chemicals that result in an induction of less than 10-fold increases over control values are termed weak EROD inducers, while chemicals that induce a 10-100 fold or >100 fold induction over control fish values are known as moderate and strong EROD inducers, respectively. In addition to *in vivo* tissue studies, EROD activity may also be monitored *in vitro* by incubating cell lines with various xenobiotic compounds to determine agonistic or antagonistic effects on EROD activity (108; 109).

2.3.2.2 Metallothionein

Metallothionein is a low molecular weight cysteine-rich intracellular protein that binds numerous heavy metals. Metallothionein has roles in the natural homeostasis of metals such as zinc and copper, but also can be used to help detoxify heavy metals such as lead or cadmium to which an organism is exposed. Due to its ability to help eliminate metals from organisms, up-regulation of metallothionein messenger ribonucleic acid (mRNA) and protein may be used as a biomarker of heavy metal exposure and environmental contamination (21; 110).

2.3.2.3 Vitellogenin

Endocrine disrupting compounds can have substantial effects on reproductive physiology in organisms such as fish, even at concentrations as low as 0.32 ng/L [EE2; (111)]. Due to the high degree of prevalence in the environment (74; 64; 42; 48) and the low concentrations required to elicit a physiological effect, numerous biomarkers for estrogenic compounds have been developed in aquatic organisms, primarily teleost fish. Vitellogenin (Vtg) is an egg yolk precursor lipoglycophosphoprotein that is naturally produced in the liver tissue of reproductively mature female teleost fish. Under normal conditions, this protein is synthesized in response to elevated endogenous levels of E2 and is transported to the ovaries via the plasma. Once at the ovaries, Vtg is incorporated into the developing oocytes by receptor-mediated uptake, after which it is subsequently broken down into various egg yolk proteins. High concentrations of Vtg are not normally detected in the plasma of immature female or male fish, but may be found in the plasma of such fish exposed to E2 or other estrogenic compounds *in vivo* (112), as the gene encoding for Vtg protein is not sex linked. Vitellogenin may be detected in whole-body

tissue extracts or in the plasma of fish using western blots and quantified using an enzyme-linked immunosorbent assay (ELISA).

2.3.2.4 Immunological assays

The immune system of an animal consists of innate and acquired immune mechanisms that protect them from invading pathogens such as bacteria, fungi or parasites. The hallmark of innate immunity, also known as natural or nonspecific immunity, is that its function does not depend upon prior exposure an infectious agent. In contrast, acquired immunity is affected by prior exposure to infectious agents. Exposure of aquatic animals, such as fish, to environmental pollutants has been shown to impact immunocompetence (6; 7), and potentially the organisms' susceptibility to disease. Xenobiotics may affect immunity through a number of mechanisms, including toxic effects of the chemical itself on immune system components, or by impacting immunity through disruption of endogenous hormone homeostasis. Many aspects of immunology are conserved between species (4), and as such, aquatic organisms may serve as potential bioindicator species for possible detrimental effects of xenobiotics on human populations. However, caution should be taken when extrapolating observed effects in one species onto that of others, such as humans, due to the complexity and diversity of different species' responsiveness to xenobiotics.

A wide range of immunological assays may be used in the assessment of aquatic pollution. Histopathology of immune tissues such as the spleen or head kidney as well as that of barrier tissues such as the gill or skin may help to determine whether xenobiotics affect tissue integrity or impact the presence of immune cells in various organs of the organism. Also, the relative numbers and proportion of different leukocytes subpopulations in the blood of fish may shed light on the effects of xenobiotic exposure on haematopoesis and the subsequent immune response elicited by exposure.

Immunostimulatory or immunosuppressive effects of chemical compounds can be observed by examining immunological parameters such as macrophage-mediated phagocytosis, chemotaxis and oxygen radical production. The levels and proportions of various immune signaling molecules or antimicrobial products such as cytokines, antibodies and anti-microbial peptides can also be measured (6; 7; 17). Each of these parameters can allow for greater insight into the effects of pollutant exposure on specific

immune responses, but may not necessarily reflect how the organism would respond when immunologically challenged. Overall immunocompetence is often determined by examining host resistance against infection, commonly a bacterial or parasitic infection (6; 17). Furthermore, pathogen challenge studies may allow more accurate and relevant ecological impacts of pollutant exposure to be predicted, due to the fact that wild organisms are constantly exposed to disease-causing pathogens in the environment.

2.4 *Use of Animal Models for the Assessment of Pollutants*

Aquatic organisms may serve as bioindicator species for pollutants and for environmental health of ecosystems due to the constant exposure of these animals to substances in the environment. Many studies have demonstrated the effects of xenobiotic exposure in aquatic teleosts and other organisms, as reviewed by (6-8). A major drawback of many of these studies is that they measure acute toxicity of chemicals, often using high concentrations (mg/L) far above that to which aquatic organisms would be exposed to in the environment. A far more likely toxicological scenario for both aquatic biota and human populations involves chronic exposure to a mixture of many xenobiotics, each of which exists at relatively low doses (μg/L) that may or may not act in a synergistic or additive manner.

The toxicology of complex mixtures of contaminants such as those found in municipal sewage effluent is of more ecological relevance. However, it is also much more difficult to determine what specific xenobiotics contribute to the observed physiological changes in test animals. Additionally, studies involving mixtures of unknown compounds are very difficult to replicate accurately, due to the variable nature of the chemical mix being released into the environment at any particular time. Vieno *et al.* (13) found significant seasonal variation in the levels of pharmaceuticals released into the environment, primarily due to a reduced elimination of pharmaceuticals by treatment processes in the winter. Even though mixtures of xenobiotics are of more relevance, it is important to note that initial studies involving the effects of individual xenobiotics on animal physiology are of great importance if a better understanding and interpretation of the effects of complex mixtures are to be made.

2.4.1 Studies on the effects of individual chemicals on fish physiology

2.4.1.1 Pharmaceutical and personal care products

Several studies have focused on the effects of individual pharmaceutical and personal care products on fish physiology. Diclofenac, a NSAID COX inhibitor commonly found in municipal wastewater effluent, has been shown to impact teleost physiology in a number of manners. Rainbow trout (*Oncorhynchus mykiss*) exposed to water-born diclofenac at concentrations ranging from 1 μg/L to 500 μg/L experienced cytological alterations in kidney, gill and liver tissue following exposure to levels as low as 1 μg/L (20). The concentrations of diclofenac used in this study potentially may be applied to effluent exposure studies, as municipal sewage treatment plant effluent can contain diclofenac concentrations of ~1 μg/L (14; 16). In the same diclofenac study (20), renal lesions and necrosis of pillar cells in the gills of trout were observed following exposure to 5 μg/L diclofenac. Furthermore, bioaccumulation of diclofenac in rainbow trout can be substantial when fish are exposed to diclofenac at environmentally relevant concentrations. Schwaiger *et al.* (19) exposed rainbow trout to 1 μg/L diclofenac and found that this pharmaceutical bioaccumulated within various tissues of the fish: as high as 2732 fold within the liver but only 69 fold in the muscle. Non-steroidal anti-inflammatory drugs can impact other aspects of animal physiology; diclofenac (108) and ketoprofen (113) have been shown to inhibit EROD activity when co-administered *in vitro* with an inducer in rainbow trout and rat hepatocytes, respectively. Exposure to salicylate, also known as salicylic acid, has been shown to impair the adrenocorticotrophic hormone mediated corticosteroidogenesis in rainbow trout interrenal tissue, as well as to reduce the transcript levels of steroidogenic acute regulatory (StAR) protein (114), a protein involved in steroid biosynthesis.

Other pharmaceutically active compounds have been examined for impacts on fish physiology. Mimeault *et al.* (115) exposed goldfish to the lipid regulator gemfibrozil at concentrations which have been detected in the environment (1.5 μg/L) and high (1500 μg/L) concentrations for 14 and 28 days. Following exposure, these fish were found to have an increased level of anti-oxidant enzyme activity, indicating that gemfibrozil exposure up-regulated the anti-oxidant defense status of the fish even though no oxidative

damage was observed (115). An earlier study by the same laboratory that exposed goldfish to gemfibrozil in the water (1.5 mg/L, 10 mg/L) for 14 days resulted in a gemfibrozil bioconcentration of greater than 100 fold, as well as a 50% reduction in plasma testosterone in exposed male fish (116). When cohort fish were injected with gemfibrozil (5 ng/g fish) rather than exposed through the water, fish had a 50% reduction in StAR mRNA levels 96 hours following injection, in addition to the previously mentioned testosterone effects (116). Similar to the impacts of gemfibrozil on oxidative stress, the anti-epileptic drug carbamazepine has also been found to increase the oxidative stress following 48 hours of exposure (0.2 mM) in primary cultured rainbow trout hepatocytes (117).

Estrogenic compounds have been well studied regarding their effects on reproductive physiology of fish (118; 119; 111; 120). Male goldfish exposed to 17β-estradiol (E2) in food or the water (1 μg/L, 10 μg/L) were found to have lower gonadosomatic indexes (GSI) than non-exposed males, as well as a reduction in milt production and the frequency and intensity of reproductive behaviour (119).

Detrimental reproductive effects do not only occur following exposure to estrogenic compounds at high concentrations. Exposure of fathead minnows (*Pimephales promelas*) to ethinylestradiol (EE2) at very low and environmentally relevant concentrations from 48 hours post-fertilization through sexual maturation resulted in significant physiological changes (111). Reduced egg fertilization success, skewed female sex ratios and decreased male secondary sexual characteristics were observed in fish exposed to EE2 at concentrations as low as 0.32 ng/L (111). The same study also observed a reduced GSI in female fathead minnows exposed to greater then 3.5 ng/L EE2 from an early age.

High doses of EE2 may result in physiological effects unrelated to sexual reproduction. Juvenile carp (*Cyprinus carpio*) exposed to either nonylphenol (1-15 μg/L), an estrogenic alkylphenol, or injected with EE2 (500 ng/g fish) and then sampled 70 days later were found to have severe anaemia compared to control fish (118). Furthermore, the fish exposed to EE2 in this experiment also developed tissue lesions in the kidney, liver and spleen.

2.4.1.2 Non-pharmaceutical and personal care product xenobiotics

The majority of aquatic toxicological studies that examine the effects of specific, known compounds on fish have focused on non-pharmaceutical chemicals, usually substances produced during or associated with industrial or chemical manufacturing. These xenobiotics can have substantial impacts on the physiology of fish, specifically the immune and endocrine system (6; 7). Other biomarkers of pollutant exposure may also be induced. For example, exposure of juvenile Atlantic cod (*Gadus morhua*) to North Sea oil, major components of which are PAHs and alkylphenols, caused a strong induction of the aryl hydrocarbon biomarker CYP1A protein, and a corresponding increase in EROD activity compared to clean water control fish (121).

Some of the most pervasive of environmental pollutants are dioxins: halogenated organic compounds formed from such processes as coal burning, diesel truck exhaust and metal smelting. Dioxins are highly toxic and can have a multitude of detrimental effects in living organisms. For example, carp captured from rivers with dioxin contamination were found to have increased CYP1A activity, as well as a reduced plasma estrogen levels and lower GSI in female fish (22).

Polychlorinated biphenyls (PCBs) are also often found in the environment; this is not surprising due to their common use in electrical transformers and capacitors. PCBs have been identified to induce immunosuppression in several teleost species (122; 4; 22; 123). For example, smallmouth bass (*Micropterus dolomieu*) from PCB contaminated environments were found to have reduced immune function compared to control fish from a reference site, illustrated by a reduced kidney phagocyte-mediated superoxide anion production, reduced phagocytosis of opsonized latex particles and a reduced level of superoxide dismutase activity (124). Another species of fish, Chinook salmon (*Oncorhynchus tshawytscha*) isolated from a PAH and PCB polluted site had reduced host resistance compared to control fish upon challenge with the bacteriium *Vibrio anguillarum* (122), likely due to a PAH/PCB induced immunosuppression. Finally, Sures and Knopf (123) exposed European eel (*Anguilla anguilla*) to various combinations of sublethal concentrations of either cadmium (~22 μg/L), PCB (100 ng/g body weight), or both chemicals and then infected the fish with the nematode parasite *Anguillicola crassus*. Seventy-six days of treatment with PCB contaminated water, either with or

without concurrent cadmium exposure, resulted in a humoral immunosuppression characterized by elimination of the eel's anti-parasite antibody response (123).

The polycyclic aromatic hydrocarbon benzo[*a*]pyrene (BaP) has also been shown to impact the immune system of teleost fish. Benzy[*a*]pyrene is produced through incomplete combustion of various substances and can be detected in automobile exhaust, tobacco smoke, burnt food and, not surprisingly, in the environment (7). Carlson *et al.* (30) injected Japanese medaka with BaP at various concentrations (2, 20 or 200 μg/g body weight) and one week later examined a number of immune parameters. The authors found that exposure of medaka to the lowest concentration used, 2 μg/g body weight, resulted in the suppression of mitogen-stimulated lymphocyte proliferation, while at higher BaP doses the number of antibody forming cells, levels of phagocyte-mediated superoxide anion production and host resistance against the bacterial pathogen *Yersinia ruckeri* were all reduced, indicative of significant BaP immunosuppression.

Pesticides make up many of the toxic compounds found in the environment, and are unique in that they are the only chemicals that are purposefully released in large quantities into the environment. Pentachlorophenol (PCP) is one such pesticide, acting as both a wood preservative and a fungicide, and has been shown to suppress the activity of immune cells from crucian carp (31). The authors exposed carp primary macrophages to between 1 mg/L and 50 mg/L PCP *in vitro* and found that mRNA expression of the key pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin 1-beta (IL-1β) were reduced in exposed macrophage cultures compared to unexposed macrophages. Additionally, supernatant from macrophage cultures can contain many immunoactive peptides, and when the supernatants from these PCP exposed macrophage cultures were exposed to B cells, the resulting B cells were less mature and secreted lower titers of immunoglobulin M (IgM) compared to controls.

Pesticides may also immunosuppress fish that are exposed *in vivo*, as demonstrated by reduced host resistance against challenge with pathogens. Japanese medaka exposed to the insecticide malathion (0.1 mg/L or 0.3 mg/L) for up to 21 days had a reduced resistance against infection when challenged with *Y. ruckeri*, likely due to a reduced number of humoral immunity antibody plaque-forming cells (29). Additionally, pesticides can affect the reproductive endocrinology of aquatic organisms.

Spanò *et al.* (26) exposed goldfish for 21 days to the common herbicide atrazine (1.0 mg/L) and found that exposed fish exhibited suppressed levels of plasma testosterone and 11-ketotestosterone, as well as elevated blood E2 concentrations and structural changes in reproductive tissues compared to control fish.

Contamination of environments with heavy metals such as mercury, lead or cadmium has been detected in numerous parts of the world and many studies have examined physiological changes that result from exposure to metals (125; 7). For example, rainbow trout (*Salmo gairdneri*) secreted excessive amounts of gill mucus following exposure to mercury compounds (126). Additionally, stress responses of heavy metal exposed fish has been demonstrated *in vitro* after exposure of red blood cells from sea bream (*Sparus sarba*) to 0.1 μM cadmium, lead, or chromium for one hour (127). The authors found that metal exposure induced significant increases in expression levels of blood heat shock protein 70, an indicator of physiological stress.

2.4.2 Studies on the effects of municipal wastewater on fish physiology

Municipal sewage treatment plant effluent contains many different xenobiotics, including pharmaceuticals and personal care products (14; 1; 2). Exposure to municipal wastewater, either in a laboratory setting or in rivers and streams located downstream of effluent outflows, can substantially affect the physiology of aquatic organisms such as freshwater fish (7; 3; 8).

Municipal wastewater can potentially contains aromatic hydrocarbons from a variety of sources such as road runoff and petroleum spills. As such, it is not surprising that studies have shown that exposure of rainbow trout to diluted tertiary treated municipal effluent results in an approximate three fold increase in liver CYP1A EROD activity, as well as a corresponding increase in PAH metabolites in the bile of these fish compared to clean water controls (128). Japanese medaka also have been shown to have an increased level of EROD activity when exposed to diluted sewage effluent (32). This indicates that aryl hydrocarbons are present in municipal treated wastewater in levels sufficient to invoke a significant increase in CYP1A catalytic activity. *In vitro* exposure of liver hepatocytes, one of the tissues with the greatest levels of CYP1A activity (104),

to municipal effluent resulted in both a significant increase in EROD activity and cytotoxic effects on the cells (117), illustrating the potentially harmful effects that treated sewage treatment plant effluent can have on fish physiology. Ma *et al.* (32) also noted that the hepatosomatic index of sewage effluent exposed fish was reduced compared to control fish, further supporting the potential cytotoxic effects of xenobiotics in the effluent that were observed *in vitro* (117).

Perhaps one of the most widely researched effects of municipal sewage treatment plant effluent is its impact on the reproductive physiology of fish. Feminization is commonly observed in male fish exposed to sewage effluent, with induction of the egg yolk precursor protein vitellogenin (Vtg) being one of the most sensitive bioindicators of estrogenic contamination (23; 129; 32; 130). For example, male rainbow trout exposed to diluted sewage treatment plant effluent had an increased concentration of Vtg in their plasma compared to clean water control male fish (130). The same Vtg induction trend was observed in fish sampled downstream of sewage treatment plants or other point sources of estrogenic contamination (112; 25; 131). This is not surprising when one takes into account the extreme sensitivity of fish to estrogens (111) and the fact that in times of drought or reduced flow rate, sewage effluent may comprise a significant proportion of the stream flow (2). Elevated levels of the female hormone 17β-estradiol have been detected in the plasma of male rainbow trout exposed to sewage treatment plant effluent (130), further linking estrogenic compounds in the aquatic environment to the Vtg induction observed in male fish.

Estrogenic contamination may affect the physiology of fish in a number of other ways. For example, the gonadosomatic index (GSI) of effluent-exposed fish has been reported to be lower in fish exposed to municipal wastewater (129; 32). Yet, this biomarker may vary depending upon the wastewater and species, as rainbow trout exposed to 15% sewage effluent had higher GSI than their clean water cohorts (130). Depending upon the level of sexual maturation at the time of exposure, irreversible feminization of reproductive tissues may occur. Sexually immature male wild roach (*Rutilus rutilus*) exposed to sewage effluent for 150 days experienced a dose dependant, but reversible, induction of Vtg and an irreversible feminization of the reproductive tracts (23). In addition, the presence of oocytes in the testes of male fish exposed to treated

municipal wastewater (129), as well as female-skewed sex ratios of wild fish caught downstream of municipal sewage treatment plants (112; 25) lend further insight to the estrogenic effects of municipal sewage on aquatic vertebrates.

The ecological ramifications of estrogenic contamination are enormous. Male fish exposed to sewage treatment plant effluent can have reduced spermatogenesis (129; 32) and reduced fecundity and fertilization rates (32). Female fish may also be impacted by sewage treatment plant effluent; the number of spawning female Japanese medaka was reported to be significantly reduced in fish exposed to 40% effluent compared to controls (32).

Sewage effluent can also impact the immune systems of teleost fish, due to the varied mix of pharmaceuticals or xenobiotics found within the effluent that may potentially affect the immune system of an organism. When challenged with a pathogen, a major component of host resistance involves the proliferation of select immune cells. Exposure of rainbow rout to diluted municipal sewage effluent for 27 days resulted in an increased mitogen-stimulated proliferation ability of peripheral blood lymphocytes compared to clean water control fish (128). Alternatively, carp exposed to river water downstream of storm sewage and municipal sewage effluent outflows for 47 days were found to have reduced lymphocyte proliferative abilities compared to fish kept in a clean river (132). It should be noted, however, that the immunosuppressive effects observed in this experiment cannot be conclusively linked to sewage treatment plant effluent, as urban runoff and industrial discharge also contributed to the aquatic pollution of the experimental river.

The number of peripheral blood leukocytes in the blood may also allow for an indirect measure of the proliferative ability of immune cells and the overall immunocompetence of the fish following exposure to wastewater or specific xenobiotics. An increased number of peripheral blood granulocytes and lymphocytes were observed in goldfish after exposure for 7 days to diluted sewage effluent, but this trend was reversed by 30 days exposure (133). Others (128) also observed reduced numbers of circulating blood lymphocytes in addition to an increased number of degrading erythrocytes, indicating an immunotoxic effect of wastewater on rainbow trout.

Many other immunological assays have been used to determine the immunocompetence of fish following exposure to municipal sewage treatment plant effluent. In wastewater exposed fish, reduced activity of phagocytic cells (133) and skin abnormalities including thinness, a reduced number of mucus cells, invasion of leukocytes into skin tissue and an increased dispersion of melanosomes into melanocyte extensions (134) have been observed. These are all characteristic indicators of stress that may potentially increase susceptibility to disease. Indeed, enhanced pathogen-induced mortality has been observed in goldfish exposed to treated sewage wastewater (133), as well as increased rates of overall mortality in non-infected fish (134). Conversely, wastewater exposure can result in non-specific activation of the immune response (135) or not affect immunological parameters such as oxidative burst or phagocytosis at all (128).

2.5 *Conclusions*

The physiological effects of individual xenobiotics or mixtures such as municipal sewage effluent on the endocrinology, immunology and other physiological systems in aquatic organisms are complex. Aquatic organisms such as fish can act as bioindicator species for water quality and, indirectly, environmental health. As water scarcity increases in the world, water quality will take on greater importance, specifically regarding the presence of pharmaceutical and personal care products or other xenobiotics in the environment. In addition to the impacts of water quality on environmental ecosystems, the potential for harmful xenobiotics to persist in the environment and accumulate in drinking water is of great interest.

Chemical analysis of wastewater samples is financially expensive and inefficient when used alone, as it may underestimate the true risk of xenobiotics to aquatic organisms or the environment by disregarding additive or synergistic effects. Aquatic organisms such as fish, however, are constantly exposed to chemicals in their environment, and, as such, can potentially act as biomonitors for xenobiotics through acute toxicity, chronic toxicity, or accumulation of contaminants in their tissues. Many aspects of the physiology of lower vertebrates such as teleost fish are shared with humans (4). Thus fish may act as sentinel species for negative effects of chemicals in wastewater

before they impact people or the environment as a whole. However, care must be taken when interpreting and applying results from one study to another, due to the many variables that may affect how an organism responds to xenobiotic exposure. Freshwater fish are usually outbred, and thus a great deal of intraspecific variability exists, in addition to considerable interspecific differences in sensitivity to xenobiotics. Water temperature, reproductive status, gender and sampling stress may all potentially influence the measured assay parameters of xenobiotic exposed organisms (24; 136), such that it may be difficult to detect any subtle chemical-induced changes.

If a better understanding of the effects of toxic chemicals on living organisms or a predictive framework for potential harmful effects of xenobiotics on the environment is to be made, a greater number of studies using a wide variety of species from different trophic levels will be required. Future studies addressing the possible risks of interactions between multiple chemicals at low concentrations and the hazards of chronic exposure to low levels of contaminants are of utmost importance if the quality of the environment and drinking water can be assured for future generations.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Fish

Goldfish (*Carassius auratus*), ~8 to 10 cm in length, were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and allowed to acclimate in the Aquatic Facility of the Department of Biological Sciences at the University of Alberta for a minimum of three weeks prior to transfer to Gold Bar Wastewater Treatment Facility. Fish were housed in fiberglass tanks with a continuous flow system and fed *ad libitum* daily with trout pellets. All fish were anesthetized by immersion in a solution of tricaine methane sulfonate (MS-222) prior to manipulation (killing, clipping, bleeding or infection with parasites).

3.2 Wastewater and Exposure Protocols

Secondary treated effluent was obtained from the Gold Bar Waste Treatment Facility, a tertiary (class IV) municipal sewage treatment plant located in Edmonton, Alberta, Canada. This plant treats wastewater from a population of ~700,000 people in the greater Edmonton area and has a daily secondary treatment capacity of 310 megalitres/day. Wastewater treatment involves pretreatment with screens and grit tanks, primary treatment with sedimentation in clarifier tanks, secondary treatment in bioreactors, and tertiary treatment using a second parallel set of bioreactors where the effluent undergoes biological nutrient reduction to reduce ammonia and phosphorus. Following UV treatment, the final treated effluent is discharged into the North Saskatchewan River. In this study, this wastewater is referred to as final effluent (FE).

At the Gold Bar Wastewater Treatment Plant, a wastewater ultrafiltration system for production of reusable water for industrial purposes was used to provide continuous ultrafiltration (UF) membrane effluent. Briefly, portions of the normal final treated effluent were diverted through a hollow fiber membrane ultrafiltration system (Zenon ZeeWeed 500; pore size 0.04 μm). This membrane filtration effectively screened out

bacteria, protozoa and any remaining particulate matter. The filter was back-pulsed every 15 minutes for 30 seconds to remove biological material accumulated on the fibers. In this study, this water is referred to as UF membrane (MF) effluent. Hypochlorite was added to the MF effluent by the wastewater treatment plant in order to prevent biological fouling of pipelines; however, due to the presence of ammonia in the wastewater the hypochlorite was converted to chloramines. Measured free and total chlorine levels in MF wastewater prior to dechlorination was ~2 mg/L during the Summer 2006 experiment.

Granular activated carbon (GAC) treatment was accomplished by passing the UF membrane (MF) wastewater through a GAC unit providing laminar flow of water (~ 1.0 L/min) and >15 minutes of residence time for contact between the GAC and the wastewater. This GAC unit was constructed of polyvinyl chloride piping (1220 mm by 150 mm outer diameter), granular activated carbon (0.1-0.3 mm particle size, lignite-based, 0.625 m^2/g; Calgon, Pittsburgh, PA), glass wool (Fisher), and polyvinyl chloride diffusers. Wastewater flow was maintained continuously for 3 months and the GAC and glass wool were replaced monthly during this time period. In this study, this water is referred to as UF membrane and GAC unit (MCF) effluent.

Final effluent and UF membrane effluent flowed into large tanks (5000 L and 10000 L for FE and MF, respectively) before being further pumped into smaller fish exposure tanks (177 L or 60L) either directly (MF) or through the GAC unit (MCF treatment). Therefore, collected wastewater samples consisted of a mix of ~2 days equivalent of flow from each treatment group. This approach reduced the effect of minute-to-minute variation in wastewater quality. Final effluent constantly flowed into the 5000 L tank, whereas MF effluent was pumped into the 10000 L tank in six ninety-minute increments, equally spaced over 24 hrs (total of 9 hrs of pumping/day). During the Spring/Summer 2005 and Winter 2006 experiments, chlorine present in wastewater was removed by pumping a concentrated sodium thiosulfate solution dissolved in domestic tap water into the head tanks (final concentration ~1 mg/L wastewater; thiosulfate anhydrous; Fisher). During the Summer 2006 experiment, a concentrated sodium thiosulfate solution was pumped at a constant rate into the 10000 L MF tank, such that the free and total chlorine concentration was less than 0.03 mg/L regardless of

concentration spikes. See Figure 3.1 for a schematic layout of the experimental tank design.

Basic water chemistry conducted daily for each experimental period included temperature, dissolved oxygen, conductivity and salinity as measured using a hand-held electronic probe (YSI Incorporated, model #85-10FT). Chlorine (free and total) was measures spectrophotometrically (Hach DR/4000U Spectrophotometer) and pH by an electronic pH meter (Accumet Research, model #AR 15).

3.3 Wastewater Exposure

Over a period of 90 days goldfish were exposed to three differentially treated sewage effluents (tertiary treatment; Gold Bar Waste Treatment Plant, Edmonton, Alberta, Canada): 1) final sewage effluent (FE); 2) UF membrane filtered (MF) final effluent; or 3) UF membrane and GAC treated (MCF) effluent. Fish were killed prior to transfer to wastewater (control) and at various time points post exposure (7, 21, 60 and 90 days), at which time points tissue and plasma samples were frozen using either dry ice or liquid nitrogen and stored at -80°C prior to analysis. Water samples were analyzed for the presence and levels of various xenobiotics by gas chromatography/mass spectrometry (Alberta Research Council. Vegreville, Alberta) at each sampling time point. A 90-day test run of the study occurred during the spring/summer of 2005 (April 21, 2005 through July 19, 2005), with a repeat of the 90 day study also occurring in the winter of 2006 (January 16, 2006 through April 17, 2006). The first parasite infection study involving *Trypanosoma danilewskyi* infected fish occurred from February through April 2006, while a second parasite infection study took place from June though August 2006.

3.4 Pesticides and Pharmaceutical Analysis

The author did not perform chemical analyses of wastewater samples. Instead, samples were sent to Alberta Research Council (a Canadian Association of Environmental Analytical Laboratories accredited laboratory; Vegreville, Alberta) for qualitative and quantitative pesticide and pharmaceutical and personal care product

(PPCP) compound analysis. During the Spring/Summer 2005 experiment wastewater samples were collected on five separate occasions: April 21, 2005, May 1, 2005, May 12, 2005, June 20, 2005 and July 19, 2005. During the Winter 2006 experiment water samples were collected on or near each of the five sampling dates: January 17, 2006, January 23, 2006, February 6, 2006, March 16, 2006 and April 18, 2006. Water samples from the Aquatics Facility of the Department of Biological Sciences, University of Alberta were collected and analyzed on April 18, 2006, in which no pesticides or pharmaceutical products were detected.

Briefly, on each sampling date, two 1 L samples of effluent were collected in acid-washed amber glass bottles (EPA Certified, Edmonton, AB) for each of the three treatments: FE, MF and MCF wastewater. There was no GAC unit testing performed on the samples taken on April 21, 2005 and this time period serves as a control. For each sample, 1.0 L was extracted with dichloromethane (VWR) at a pH less than 2 using a separatory funnel. Twenty-five microliters of working standard surrogate (deuterated standards) solution was added prior to the extraction, for monitoring of losses that could occur through sample handling and to minimize the possibility of false negative results. The organic extract was dried with acidified sodium sulphate (Fisher), concentrated to ~0.5 mL on a TurboVap evaporator tube with a water bath set to 35°C. Extract was methylated with 1 mL diazomethane. The methylated sample extract was re-concentrated to a volume of less than 250 µL (to drive off excess diazomethane) using a gentle stream of nitrogen. The final sample extract volume was then increased to 250 µL via the addition of dichloromethane and 5 µL of a 20 ng/µL working internal standard (deuterated standards) solution prior to analysis by gas chromatography/mass spectrometry (Varian Gas Chromatograph, model #CP3800; Ion Trap Mass Spectrometer, model #Saturn 2200) for qualitative and quantitative determination of pesticides and residual pharmaceuticals.

A qualitative screening was performed using the relative retention time and relative abundances of two or more characteristic ions. A full identification of pharmaceuticals and pesticides screened in this manner was performed using full reference spectra. Quantitative analyzes of compounds were performed using a multi-internal standards technique in which the extracted areas of characteristic ions were

utilized in the calculation. The instrument was calibrated with a multi-point calibration curve using analytical standards in their linear range. Standards were purchased from suppliers (AccuStandard, USA) as certified standards. Compounds were identified using a relative retention time and two or more ions. Quantification was done using an ion that had been demonstrated to be free of interferences and was compared to the same ion in the standard. Values were not corrected for surrogate or method recoveries.

3.5 Cytochrome P450 (CYP1A)

Goldfish were killed prior to transfer to wastewater (control) or at various time points post exposure (Days 7, 21, 60, 90). At each sampling time point with the exception of control, liver tissue was isolated from fish exposed to FE, MF or MCF treated sewage effluent. In the Spring/Summer 2005 experiment, liver tissues were frozen using dry ice, whereas in the Winter 2006 experiment tissues were flash frozen using liquid nitrogen. Tissues were transported to the University of Alberta on dry ice prior to storage at -80°C.

3.5.1 Microsome isolation

Liver samples, frozen at –80°C, were homogenized on ice using Dounce tissue grinders and homogenizing buffer [50 mM tris[hydroxymethyl]aminomethane (Tris; Sigma), 1 mM ethylenedinitrilo)-tetraacetic acid, disodium salt dihydrate (EDTA; EMD Chemicals), 2 mM phenylmethyl-sulfonyl fluoride (PMSF; Sigma) and 2.5 mM dithiothreitol (DTT); PMSF and DTT were added just prior to use]. The homogenate was centrifuged at 4°C for 20 minutes at 12 000 g, after which the supernatant was removed using a Pasteur's pipette and subsequently centrifuged at 100 000 g for 1 hour at 4°C. The resulting microsomal pellet was resuspended in resuspension buffer (50 mM Tris, 1mM EDTA, 2 mM PMSF, 2.5 mM DTT and 20% v:v glycerol) at a 1:1 weight to volume ratio. This microsomal suspension was aliquoted and frozen at –80°C until use in the EROD assay. The homogenate and buffer solutions were kept on ice at all times.

3.5.2 EROD and protein assay

Resorufin standards (12.5 nM-248.8 nM; Sigma), protein standards (bovine serum albumin; 149.3 µg/mL–1194.0 µg/mL; Sigma) and blanks were set up in a 96-well plate. All dilutions were made using Tris Resuspension Buffer (50mM Tris in Milli-Q water,

pH 7.4) from either the dry weight of the protein or from a stock solution of resorufin (5 mM in methanol, stored at -20°C). Twelve microliters of microsome solution as well as 30 µL of a 9.5 µM 7-ethoxyresorufin solution (7-Ethoxy-3H-phenoxazine-3-one; Sigma) were added in triplicate to sample wells and the plate was incubated at 25°C for 10 minutes (fmax fluorescence microplate reader, Molecular Devices). The reaction was started by adding 30 µL of reduced nicotinamide adenine dinucleotide phosphate (NADPH; 5.4 mM in Tris resuspension buffer; Sigma) and followed by another 10 min incubation at 25°C. The reaction was stopped by the addition of 60 µL fluorescamine in acetonitrile (2.2 mM; Sigma), after which the plate was immediately read in a fluorescence plate reader for resorufin production (excitation wavelength 544 nm; emission wavelength 590 nm) and protein content (excitation wavelength 355 nm; emission wavelength 538 nm).

3.6 Vitellogenin

3.6.1 Plasma isolation

Plasma samples were obtained from male goldfish either prior to wastewater exposure (control) or following exposed to the different wastewater treatments for various time points post exposure (Days 7, 21, 60, 90). On each sampling date fish were anesthetized by immersion in a solution of tricaine methane sulfonate (MS-222) and their blood was collected from the caudal vein using cold, heparinized and aprotinated (2 trypsin inhibitory units (TIU)/mL; Sigma) 1mL syringe fitted with a 27 ½ gauge needle. The blood was immediately transferred to a vial containing an equal volume of ice cold sodium aprotinin solution (0.09% NaCl, 2 TIU/mL), and centrifuged at 3000 g for 10 minutes. The plasma was collected, aliquoted and frozen in liquid nitrogen prior to transfer to the University of Alberta on dry ice and stored at –80°C prior to use in the assays.

3.6.2 Vitellogenin assay

Vitellogenin (Vtg) was quantified by using pre-coated carp Vtg ELISA plates (Carp Vitellogenin ELISA Kit, Biosense Prod. No. V01003402). Briefly, 100 µL dilution buffer (phosphate buffered saline, 1% bovine serum albumin) was added to

nonspecific binding wells and an equal volume of plasma sample (thawed on ice; 1:5000 dilution in dilution buffer) was added to the sample wells in the pre-coated plate. The plate was incubated for 1 hr at 37°C, then each well was washed 3 times with 300µL washing buffer (phosphate buffered saline, 0.05% Tween 20). One hundred microliters of diluted detecting antibody (1:500 dilution of stock antibody solution using dilution buffer) was then added to all wells and the plate incubated at 37°C for one hour. Plates were washed three times with 300 µL of washing buffer, and then 100 µL of the kit supplied secondary antibody was added to each well (1:2000 dilution of stock solution using dilution buffer). The plate was then once again incubated for 1 hr at 37°C and then washed 5 times (300 µL washing buffer/well). Following wash with the washing buffer, 100 µL of pre-supplied substrate solution (urea hydrogen peroxide and O-phenylenediamine in dH_2O) was added to each well. The plate was incubated in the dark at room temperature for 30 min prior to stopping the reaction with 50 µL of 2M H_2SO_4. Absorbance was read 5 min later at 492 nm using a microplate autoreader (Bio-Tek Instruments).

3.7 Proliferation of Peripheral Blood Leukocytes

3.7.1 Isolation of peripheral blood leukocytes

Peripheral blood was collected by exsanguinations of anesthetized goldfish prior to exposure (control) or after 7, 21, 60, or 90 days to FE, MF or MCF wastewater using sterile 1 mL syringe fitted with a 27 ½ gauge needle. Peripheral blood leukocytes (PBL) were isolated from the blood of goldfish using procedures described by Neumann and Belosevic (137). Briefly, 0.5 mL of blood from each fish was immediately mixed with 10 mL homogenizing solution in 15 mL conical tubes and placed on ice. Homogenizing solution contained 50 µg/mL gentamicin, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 U/mL heparin in incomplete medium (MGFL-15). The blood-homogenization solution mixture was layered on 3 mL of a 51% Percoll solution (Sigma) and spun at 400 g for 20 minutes. The buffy coat was removed, washed with 10 mL of incomplete medium and then centrifuged (300 g, 10 minutes). Leukocytes were resuspended in approximately 0.5 mL of complete (CMGFL-15) medium, enumerated and diluted to 2.0

$\times 10^6$ cells/mL. Incomplete and complete medium were made as described in Neumann and Belosevic (137).

3.7.2 BrdU proliferation assay

The proliferative ability of peripheral blood leukocytes was determined using the colourimetric BrdU Cell Proliferation ELISA kit (Roche). This assay allows the quantification of cell proliferation based upon the degree of incorporation of 5-bromo-2-deoxyuridine (BrdU; an analogue of thymidine) into the DNA of cells. Equal numbers of PBL were seeded in sterile 96 well plates (50,000 cells/well) in addition to either complete medium alone (non-stimulated) or an equal volume of mitogens (10 μg/mL concanalvin A, ConA; 10 ng/mL phorbol 12-myristate 13-acetate, PMA; 100 ng/mL calcium ionophore A23187) and cultured for 48 hours at 20°C. BrdU labeling reagent then added to a final concentration of 20 μM and the plates incubated for a further 24 hours at 20°C. The plates were centrifuged (300 g, 10 minutes), the supernatant removed and the pellet dried (60°C, 1 hour). Two hundred microliters of FixDenat solution was added to each well and the plate incubated for 30 minutes at 20°C. One hundred microliters of ELISA blocking buffer (1% bovine serum albumen in phosphate buffered saline and 0.1% sodium azide) were added following the removal of FixDenat. One hour later the ELISA blocking buffer was removed and 100 μL/well of anti-BrdU POD working solution was added to each well and incubated (90 minutes; 20°C). The antibody conjugate was removed and the plates washed in triplicate with 200 μL/well of washing solution. Substrate solution (100 μL/well) was added and the plate allowed to incubate for 10-15 minutes prior to stopping the reaction with 2M H_2SO_4 (25 μL/well). Absorbance was measured at 450 nm using a microplate reader (Bio-Tek Instruments) within 5 minutes of acid addition.

3.8 RT-PCR Analysis of Goldfish Kidney CFS1-R, Granulin, TNF-alpha, and TLR-22 expression

Six male and six female fish from each effluent treatment group were removed either prior to wastewater exposure (control) or on Days 7, 21, 60 or 90 after exposure. For each time point, fish were anesthetized with MS-222 and killed by cervical

dislocation. The kidneys were aseptically removed, placed in cryotubes and immediately frozen in liquid nitrogen. The kidneys were transported to the university on dry ice and then placed in a -80°C freezer until RNA isolation.

Total RNA was isolated from goldfish kidneys using TRIzol® RNA Extraction Reagent (Invitrogen) as per the manufacturer's directions. First-strand synthesis of cDNA was done using random hexamers primers (Invitrogen) and SuperScript™ II RT (Invitrogen) with 5 μg of total RNA according to manufacturer's protocols. The primers and PCR cycling parameters used to amplify goldfish specific genes by RT-PCR were:

CSF-1R (sense 5'-GGCAGCACAAGAACATCGTCAAC-3';
antisense 5'-GGCGTCCAGGTTCCAGCACATC-3');
94°C for 3 min; 29 cycles of 94°C for 20 s; 60°C for 20 s; 72°C for 2 min. A final extension of 72°C for 10 min was added following cycling.

Granulin (sense 5'-AAGATGGTTCCAGTGTTGATGTTAC-3';
antisense 5'-ACCCCACTGGCCGGCTGCTGT-3');
94°C for 3 min; 23 cycles of 94°C for 20 s; 60°C for 20 s; 72°C for 2 min. A final extension of 72°C for 10 min was added following cycling.

TNF-alpha (sense 5'-TGCATATGACCCTGACGTGTG-3';
antisense 5'-GCCGCTCCGAGGTAAATGGTG-3');
94°C for 3 min; 28 cycles of 94°C for 30 s; 56°C for 30 s; 72°C for 2 min. A final extension of 72°C for 10 min was added following cycling.

TLR-22 (sense 5'-TTTCAGGATTCCGTCGATACC-3';
antisense 5'-GTTCGTTTCTTCACCAGCTTCC-3');
94°C for 3 min; 29 cycles of 94°C for 35 s; 60°C for 35 s; 72°C for 2 min. A final extension of 72°C for 10 min was added following cycling.

Beta-actin (sense 5'-GCACGCGACTGACACTGAAG-3';
antisense 5'-GAAGGCCGCTCCGAGGTA-3');
94°C for 3 min; 25 cycles of 94°C for 35 s; 60°C for 35 s; 72°C for 2 min. A final extension of 72°C for 10 min was added following cycling.

Each reaction contained 5 pmol of each amplification primer, 1 mM dNTPs, 10× PGR buffer (1.5mM $MgCl_2$, 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.5 units *Taq* Polymerase and 0.5 µl of cDNA template. Immediately following PCR amplification, 10 µl of the PCR samples were run on a 1.5% agarose/TAE gel, stained using ethidium bromide, and photographed on a UV transluminator (Fisher Biotek, FBTIV-88). Relative expression levels of the specific gene bands were determined by densitometry using the Kodak ID 3.0 software (Eastman Kodak).

3.9 Parasite Infection Model

Trypanosoma danilewskyi parasites were originally isolated in 1977 from a wild crucian carp (*C. crassius)* by J. Lom. Parasites were obtained from P.T.K. Woo, University of Guelph, Ontario, and initially maintained through infection of naïve goldfish with trypanosomes isolated from infected fish prior to development of an *in vitro* culture method using the trypanosome growth medium (TDL-15) and 10% heat inactivated sterile goldfish serum [as described in (138; 139)]. Parasites were passaged approximately every 5-7 days. Fish were acclimated to their tanks for at least one week prior to infection with *T. danilewskyi*. Before infection of the fish, a small blood sample was withdrawn and collected in heparinized microcapillary tubes from each fish in order to ensure the fish did not have any pre-existing haematoflagellate infections. Fish were fin clipped to identify individual fish for future monitoring and injected intraperitoneally with either ~ 6.25×10^6 parasites/fish or with ~1.0×10^7 parasites/fish using a 26 G needle and syringe. Previous observations (139) determined that inoculation of fish with 6.25×10^6 parasites/fish resulted in all fish developing infections and low mortality. The inoculation dose of 1.0×10^7 parasites/fish was chosen as we wished to challenge the fish

with a greater dose of parasites, but not to the degree where very high mortality rates would occur.

The parasite infection experiments were preformed on two separate occasions. The first parasite infection experiment was done between February 5, 2006 and April 30, 2006 and involved the infection of control goldfish and goldfish exposed to municipal wastewater for twenty days with 6.25×10^6 parasites per fish. The second parasite infection experiment involved the infection of control goldfish and fish that had been exposed to wastewater for approximately seven days with either 6.25×10^6 parasites per fish or a higher dose of 1.0×10^7 parasites per fish and occurred between June 1, 2006 and August 24, 2006.

Parasitemias in individual fish were determined three days post infection, and then weekly thereafter for 12 weeks. Briefly, a small amount of blood was diluted in tri-sodium citrate anticoagulant (100 mM tri-sodium citrate, 40mM glucose, pH 7.3) and parasites enumerated using a haemocytometer and bright-field microscopy. For fish with low parasitemia, average parasites/mL blood was determined by enumerating parasites located at the packed cell-plasma interface of spun, heparinized capillary tubes.

3.10 Statistical Analyses

For statistical analysis, all data were log transformed prior to analysis in order to ensure a similar degree of variation between experimental groups and to satisfy the requirements of the statistical test. Regarding Vtg analysis, the average Vtg concentration (μg/mL blood) for each male fish was first determined using a carp Vtg standard curve (linear standard curve $R^2 = 0.9948$, $y = 0.0343x + 0.0121$). Following this, the lowest individual fish Vtg concentration was then subtracted from all values such that the lowest fish Vtg concentration was equal to zero. Subsequently, each fish's Vtg concentration was log transformed ($\log(x+1)$) prior to statistical analysis. For the mitogen-stimulated PBL proliferation data, a proliferation ratio was determined for each fish by dividing the mean mitogen-stimulated proliferation absorbance by the appropriate non-stimulated proliferation absorbance value. This proliferation ratio was then log transformed in order to reduce variation amongst experimental groups. Following this

transformation, a factoral analysis of variance (ANOVA) test was preformed for each data set followed by a Tukey multiple comparisons post-hoc test to examine (when appropriate) the effect of the wastewater treatment, the length of exposure, the sex of the fish, and the year in which the experiment was conducted on the appropriate responding variable. In addition, data were analysed using a one-way ANOVA (Dunnett's multiple comparison test) to determine significant difference of wastewater treatment experimental groups compared to control fish. For *T. danilewskyi* parasite infection studies, statistical analysis used log transformed parasitemia data and the SAS system (Mixed Procedure) analysis to examine a variety of parameters in either the Winter 2006 experiment or the Summer 2006 experiment. For all statistical tests, significance was indicated by a P value of < 0.05. No statistical analysis was done on the xenobiotic data, due to the small sample size ($n = 2$ for each sampling day wastewater treatment).

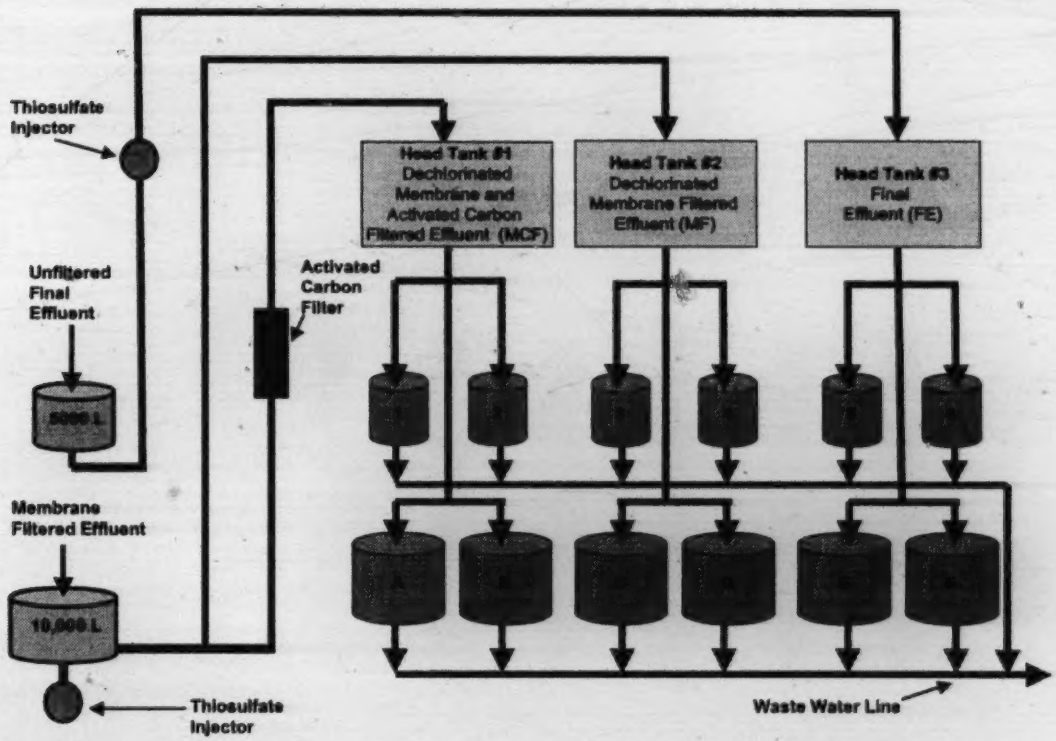
Thiosulfate Injector
Head Tank #1 Dechlorinated Membrane and Activated Carbon Filtered Effluent (MCF)
Head Tank #2 Dechlorinated Membrane Filtered Effluent (MF)
Head Tank #3 Final Effluent (FE)
Unfiltered Final Effluent
Activated Carbon Filter
5000 L
Membrane Filtered Effluent
10,000 L
Thiosulfate Injector
Waste Water Line

Figure 3.1 Schematic drawing of experimental set-up at Gold Bar Wastewater Treatment Plant. Exposure tanks 1-6 contained 60 L of wastewater effluent (MCF, MF or FE) and were used for the parasite challenge experiments, whereas exposure tanks A-F held 177 L of wastewater and contained goldfish used for all other assays. Flow rate was ~0.5 L/min for tanks 1-6 and ~1.0 L/min for tanks A-F.

CHAPTER FOUR

CHEMICAL ANALYSIS OF XENOBIOTICS IN MUNICIPAL WASTEWATER

4.1 Introduction

Xenobiotics have been commonly detected in municipal wastewater, both prior to and following treatment (1; 2; 67; 3; 8). In large municipalities, pharmaceuticals and personal care products can enter wastewater through metabolic excretion and disposal of unused medication in toilets, whereas pesticides may enter wastewater through street or landscape runoff. Xenobiotic concentrations in municipal sewage effluent depend on both biotic and abiotic processes, such as the volume of effluent treated, the amount of drugs being taken by the population, treatment type (140), sludge retention time (69) and the treatment temperature of the sewage (13). In the body, xenobiotics may associate with polar molecules in order to enhance excretion. The concentrations of a xenobiotic may increase in the effluent following wastewater treatment, likely due to cleavage of the chemicals from molecules such as glucuronides, sulphates or sulpho-glucuronides (57).

In the sewage treatment plant, xenobiotics are removed primarily by biodegradation and sorption (2); however, the percent removal of the compounds was found to be highly variable (65; 69; 49). Passage of treated wastewater through a granular activated carbon (GAC) unit is known to be highly effective in removing chemical contamination from a wide variety of fluids, and is commonly used in hospitals, industrial or commercial operations, and households to increase water quality. For example, GAC obtained from six to twelve month old water purifiers has been found to successfully reduce the concentration of the herbicide atrazine from contaminated wastewater (141). A wide variety of raw materials can be used in the manufacturing of activated carbon, such as coal, wood, peat, coconut shells and petroleum coke. During treatment of water with activated carbon, the particles adsorb molecules by fixing them to carbon atoms by the London dispersion force, a form of van der Waals force.

Despite the well-known ability of granular activated carbon (GAC) to adsorb pollutants, it is not commonly used to remove contaminants from final treated effluent in

wastewater treatment plants. Wastewater treatment plants can process hundreds of thousands of litres of wastewater daily, in which the final effluent may have several mg/L of suspended particulate matter. Normally, this particulate matter would quickly foul the GAC unit, greatly reducing the surface area of the GAC particles. This would result in a greatly reduced efficacy and longevity of the GAC unit. Through use of both an ultrafiltration (UF) membrane to remove particulate matter and subsequent GAC treatment to reduce most organic xenobiotics, safer reusable water may be efficiently produced on the scale of a large municipal wastewater treatment plant.

Due to the increased prevalence of UF membrane treated sewage effluents as a potential reusable water source, I examined the efficacy of a commercial UF membrane in the removal of xenobiotics from final treated sewage effluent. Secondly, I examined the efficacy of a subsequent GAC unit for the removal of various xenobiotics from the UF membrane treated effluent. Concentrations of a number of representative pesticides and pharmaceuticals were determined using gas chromatography and mass spectrometry in reuse water, and compared to those found in the source final sewage effluent for both the Spring/Summer 2005 and Winter 2006 experiments. No statistical analysis was done on the xenobiotic data, due to the small sample size (n = 2 for each wastewater sample analyzed on each sampling day).

4.2 Results

4.2.1 Daily water chemistry

Table 4.1 and Table 4.2 show the basic daily water chemistry analysis and flow rates found in each of the treatment groups for both Spring/Summer 2005 and Winter 2006 experiments, respectively. The basic water chemistry for the June-August 2006 parasite study (Summer 2006) is shown in Table 4.3. There were no discernable differences in reuse water chemistry between either the final effluent (FE) or UF membrane treatment groups (MF and MCF). During the Winter 2006 study, the Gold Bar Wastewater Treatment Facility Laboratory analyzed ammonia levels in the fish tanks on a daily basis (automated colourimetric phenate method, MDL = 0.005 mg/L NH_3). During this time, mean ammonia levels of 8.47 ± 0.49 mg/L, 1.18 ± 0.15 mg/L, and 0.91

± 0.14 mg/L nitrogen were detected in FE, MF and MCF wastewater, respectively. While the range of ammonia in the reuse water was fairly low (0.27 – 3.79 mg/L for MF wastewater and 0.16 – 2.72 mg/L for MCF wastewater), ammonia levels were found to spike in FE wastewater as high as 14.15 mg/L (the range of detected NH_3 in FE wastewater was 3.60 – 14.15 mg/L). At times, the concentration of ammonia in FE fish tanks remained greater than 9.0 mg/L for at least eight days (January 29 to January 26, 2006; data not shown).

4.2.2 Analysis of pesticides in wastewater

In each of the three treatment groups (FE, ME and MCF), wastewater samples were analyzed at various time points throughout the Spring/Summer 2005 and Winter 2006 experiments. All water samples were analyzed for a panel of sixty-eight pesticides, of which eight were detected in the municipal wastewaters (Table 4.4). Figure 4.1 represents concentration of the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) found in FE, MF and MCF wastewater in the Spring/Summer 2005 experiment. At the beginning of the wastewater treatment process (April 21, 2005), concentrations of 2,4-D were relatively low in all treatment groups (0.026 µg/L and 0.027 µg/L for FE and MF wastewater, respectively; the GAC filter was not functioning at this time). At later time points however, the mean concentration of 2.4-D had risen substantially, peaking at 0.283 µg/L in FE wastewater (July 19, 2005) and 0.297 µg/L in MF wastewater (May 12, 2005). UF membrane filtration (MF wastewater) did not substantially reduce the concentration of 2,4-D (<10 %). However, GAC treatment was effective in reducing 2,4-D in MCF wastewater, with values found as low as 0.040 µg/L (July 19, 2005; Fig. 4.1). The reduction efficiency of 2,4-D by activated carbon ranged between 44% (May 12, 2005) and 85% (July 19, 2005), while at low concentrations little reduction occurred (May1, 2005; Fig. 4.1).

Not surprisingly, 2,4-D was detected at relatively low levels in all treatment groups in the Winter 2006 experiment (Fig. 4.2). This herbicide was not detected in any wastewater treatments on January 17, 2006, Jan 23, 2006 or March 16, 2006. On February 6, 2006 2,4-D was detected at levels of 0.060 µg/L in MF wastewater alone. Since this chemical was not detected in FE wastewater at this time point, the source

effluent for MF wastewater, it was likely that the 2,4-D values on February 6, 2006 were not representative of actual herbicide levels in the wastewater. This conclusion was also supported by the fact that the date of collection of this sample, early February, was mid-to-late winter in Edmonton, Alberta, and no pesticide application would have been occurring at this time. At the last sampling point, April 18, 2006, low levels of 2,4-D were detected in all wastewater treatment groups (0.020 µg/L, 0.036 µg/L and 0.006 µg/L for FE, MF and MCF wastewater, respectively). These concentrations were similar to those detected in the Spring/Summer 2005 experiment at the same time of year (April 21, 2005). Activated carbon filtration reduced the levels of 2,4-D in the MCF wastewater samples, with a removal efficiency of 83% compared to MF wastewater, whereas the concentration of 2,4-D in MF wastewater was similar to that of FE wastewater.

Another herbicide, MCPP (2-(2-methyl-4-chlorophenoxy) propionic acid), was also detected in municipal wastewater in the spring and summer of 2005 (Fig. 4.3). On April 21, 2005 the concentrations of MCPP in effluent of FE and MF wastewater were 0.018 µg/L and 0.020 µg/L, respectively (values for MCF wastewater were not found as the GAC unit was not functioning at this time). At later sampling time points, the measured concentration of MCPP increased, reaching peak levels of 0.175 µg/L, 0.176 µg/L, 0.081 µg/L for FE, MF and MCF wastewater, respectively, on June 20, 2005. The removal efficiency for MCPP was less than 5% in MF wastewater compared to FE wastewater. However, the reduction by activated carbon ranged from 31% (May 12, 2005) to 75% (July 19, 2005) of the measured MCPP. In the Winter 2006 experiment, MCPP was detected at high concentrations only at the last sampling time point, April 18, 2006. Much higher levels of this herbicide were detected in all wastewater effluent samples on this date compared to the levels found on April 21, 2005, indicating a year-to-year variability in the detection or possible application of MCPP (Fig. 4.4). Higher levels of MCPP were detected in MF wastewater and FE wastewater on April 18, 2006 - 0.78 µg/L and 0.55 µg/L, respectively, while activated carbon filtration reduced the herbicide concentration in MCF wastewater by 56% compared to MF wastewater (0.34 µg/L). The increase of 2,4-D and MCPP in late spring and early summer in both 2005 and 2006 was expected due to standard household and commercial application of these agricultural chemicals during these seasons (142).

Other pesticides were also detected in the effluent during the Spring/Summer 2005 (Table 4.5-4.7) and Winter 2006 experiments (Table 4.8-4.10). For example, in the Spring/Summer of 2005 experiment 2,4-dichlorophenol was removed (>99%) by carbon treatment (MCF wastewater) but not removed in MF wastewater (July 19, 2005). Similarly, dicamba was removed by 48% in MCF wastewater but not removed in MF wastewater. Some of the herbicides and pesticides were not detected in certain sampling periods; bromacil, triclopyr, and diazinon were only detected on June 20, 2005. Regarding the Winter 2006 experiment, the levels of 2,4-dichlorphenol were greatly reduced compared to the Spring/Summer 2005 experiment (Table 4.5-4.10). The removal efficiency of 2,4-dichlorphenol in MF wastewater was 39%, but subsequent activated carbon filtration once again reduced the concentration of this compound to below method detection limits in MCF wastewater. Similar to the Spring/Summer 2005 experiment, dicamba levels were not substantially reduced following membrane filtration; however, in the Winter 2006 experiment activated carbon filtration efficiently removed dicamba in the wastewater (>99%).

4.2.3 Pharmaceuticals and personal care products

Pharmaceuticals and personal care products were also analyzed at the same time points in the same water samples. Of the eighteen compounds screened, ten were detected in the wastewater effluents and are listed in Table 4.4.

4.2.3.1 Non-steroidal anti-inflammatory drugs

Figure 4.5 represents the concentration of salicylic acid, a deacetylated form of the analgesic acetylsalicylic acid, detected during the Spring/Summer 2005 experiment. Concentrations of salicylic acid increased significantly in FE and MF wastewater on July 19, 2005, compared to 0.03 µg/L on April 21, 2005. The removal efficiency of salicylic acid was up to 15% by the UF membrane (MF wastewater), and it varied considerably in the MCF wastewater. At low salicylic acid levels removal by activated carbon (MCF wastewater) was similar to that by membrane UF (MF wastewater), but once the levels rose (July 19, 2005), GAC removal efficiency increased to 75%. During the Winter 2006 experiment, salicylic acid was not detected in wastewater samples in a consistent manner. Low levels of this compound were detected in water samples on January 17, 2006,

January 23, 2006 and March 16, 2006, but salicylic acid was not observed in any wastewater samples on February 6, 2006 or April 18, 2006 (Fig. 4.6). On January 17, 2006, salicylic acid was only detected in the MCF wastewater (0.036 μg/L) and not in FE or MF wastewater. Due to the close proximity of measured values to the method detection limit (0.025 μg/L) and the absence of this compound in both the FE and MF wastewater, it is possible that the salicylic acid detected may not be representative of the actual concentration of this compound in GAC filtered wastewater. On January 23, 2006 neither membrane UF nor GAC filtration reduced the low salicylic acid concentration in the effluent (Fig. 4.6). On June 20, 2005, similar concentrations and low salicylic acid removal efficiency were observed in MF and MCF wastewater (Fig. 4.5). Salicylic acid levels rose to a peak of 0.072 μg/L in FE wastewater on March 16, 2006. On this date, as observed on July 19, 2005, membrane UF did not substantially reduce salicylic acid concentration in MF wastewater compared to FE wastewater. In contrast to the ~75% reduction GAC efficiency observed in 2005, subsequent carbon filtration reduced salicylic acid levels by only 36% compared to FE wastewater on March 16, 2006. This variability in removal efficiency may have been influenced by the age of the carbon filter or interactions with unknown chemicals in the GAC filtration unit at different times.

Ibuprofen was another analgesic detected in the municipal wastewater during both experiments. In the Spring/Summer 2005 experiment, relatively low levels of ibuprofen (~0.100 μg/L in FE and MF wastewater) were detected for the majority of sampling time points. The highest concentration of ibuprofen was found in the FE and MF wastewater on May 12, 2005 (0.426 μg/L and 0.493 μg/L for FE and MF wastewater, respectively, Fig. 4.7). On July 19, 2005, MCF treatment removed almost 100% of the ibuprofen from the MF wastewater. Alternatively, when ibuprofen concentration was low (0.017 μg/L, June 20, 2005) the efficacy of activated carbon treatment was low with only 25% of the ibuprofen removed in MCF wastewater. When samples were taken during the winter of 2006, much higher levels of ibuprofen were detected in the FE wastewater (2.07 μg/L on January 23, 2006, and 1.21 μg/L on February 6, 2006, Fig. 4.8). At all other sampling points, lower levels of this analgesic were observed in the FE. Ultrafiltration successfully eliminated ibuprofen in MF wastewater during the Winter 2006 experiment, resulting in a removal efficiency of greater than 88% when ibuprofen

was present in high amounts and 32% when the chemical was only found in low concentrations. Activated carbon filtration successfully removed greater than 95% of the ibuprofen in MCF wastewater at the first three Winter 2006 sampling points. At later time points, as observed in the Spring/Summer 2005 experiment, GAC removal efficacy was reduced to ~70% when the ibuprofen concentration was low in FE wastewater.

The removal efficiency of diclofenac was shown in Fig. 4.9 and Fig. 4.10 for the Spring/Summer 2005 experiment and Winter 2006 experiment, respectively. During the Spring/Summer 2005 experiment, there was substantial removal of diclofenac in MCF wastewater on June 20, 2005, and July 19, 2005 (61% and 79%, respectively), but this high removal efficiency was not observed in the May 1, 2005 and May 12, 2005 wastewater samples (Fig. 4.9). Membrane UF did not substantially reduce diclofenac concentrations (<15%; MF wastewater) during the Spring/Summer 2005 experiment. In the Winter 2006 experiment, substantially higher concentrations of diclofenac were detected in the FE and MF wastewater samples: up to 2.3 μg/L (Fig. 4.10) compared to the maximum of 1.0 μg/L detected on April 21, 2005 (Fig. 4.9). Activated carbon filtration eliminated diclofenac in the wastewater samples by as much as 97%; however, this efficacy was variable depending upon sampling date and was as low as 40%. The UF membrane did not remove diclofenac from final effluent; in fact, elevated concentrations of diclofenac were consistently detected in MF wastewater samples at each Winter 2006 sampling time point (Fig. 4.10).

The NSAID naproxen was also detected in the wastewater effluents at levels ranging as low as 0.01 μg/L in MCF wastewater (July 19, 2005) to 0.44 μg/L in FE wastewater (January 23, 2006). During the Spring/Summer 2005 experiment, low removal efficiency was observed by UF membrane treatment (Fig. 4.11; MF wastewater). The UF membrane only removed a maximum of 20% of the naproxen (June 20, 2005) while the removal by activated carbon ranged up to 90% (July 19, 2005; Fig. 4.11). Higher peak concentrations of naproxen were detected in the Winter 2006 experiment compared to the Spring/Summer 2005 experiment (Fig. 4.12). When naproxen levels were high (January 23, 2006), membrane filtration resulted in high removal efficiency (71%). However, this removal efficiency in MF wastewater was reduced at lower naproxen concentrations; removal efficacy ranged between 27% and 63% (Fig. 4.12).

Activated carbon filtration resulted in greater than 88% removal of naproxen in MCF wastewater during the Winter 2006 experiment, regardless of the initial concentration of naproxen in the FE wastewater.

4.2.3.2 Anti-epileptic Drugs

The anti-epileptic and anti-mania medication carbamazepine is commonly found in municipal wastewater (14), and was detected in both the Spring/Summer 2005 (Fig. 4.13) and Winter 2006 (Fig. 4.14) experiments at concentrations as high as 2.3 μg/L (Jan 23, 2006; FE wastewater). In general, less than 20% of this pharmaceutical was removed by the UF membrane (MF wastewater), while activated carbon treatment eliminated between 55% to 100% of carbamazepine in MCF wastewater (Fig. 4.13; Fig. 4.14). Variable levels of this compound were found in the municipal wastewater. For example, high concentrations of carbamazepine were detected in both FE and MF wastewater on January 17, 2006, and January 23, 2006; while on May 1, 2005, February 6, 2006, and March 16, 2006, this compound was not detected in wastewater. Also, the concentration of this xenobiotic varied by as much as 2.3-fold in FE, depending upon the sampling date (Fig. 4.14). Reasons for these fluctuations are unknown.

4.2.3.3 Lipid Regulating Drugs

Gemfibrozil, a cholesterol-lowering drug detected in the municipal sewage effluent, was removed 70% to 100% by activated carbon in the Winter 2006 study (Fig. 4.16), or between 20-90% during the Spring/Summer 2005 experiment, depending upon the sampling date (Fig. 4.15). Membrane UF treatment removed variable levels of gemfibrozil in MF wastewater; during the Spring/Summer 2005 experiment, removal efficiency by the UF membrane ranged up to 40% (Fig. 4.15; MF wastewater). However, during the Winter 2006 experiment membrane UF did not result in substantial removal of this xenobiotic (Fig. 4.16). Gemfibrozil levels in FE wastewater ranged between 0.12 μg/L and 0.30 μg/L. In addition to gemfibrozil, another lipid regulating drug, bezafibrate, was also detected in wastewater on April 21, 2005, July 19, 2005 and April 18, 2006 (Table 4.5-4.10). The UF membrane did not significantly reduce the concentration of this chemical in MF wastewater, but subsequent activated carbon

filtration resulted in a removal efficiency of 65%-100% (MCF wastewater; Winter 2006 and Spring/Summer 2005 experiments, respectively).

4.2.4 Other Xenobiotics

The antibacterial and antifungal agent triclosan, and the triclosan metabolite methyl triclosan, were detected in FE and MF wastewater at various sampling points (Table 4.5-4.10). These compounds are common components of such items as anti-bacterial soaps and other consumer products. In the case of the Spring/Summer 2005 experiment, the UF membrane was not effective at removing these compounds (Table 4.5; MF wastewater). However, in the Winter 2006 experiment triclosan was completely removed from the MF wastewater (Table 4.6-4.8). These components were removed to below detectable limits in MCF wastewater at each sampling time point. It should be noted that in order to volatilize triclosan for GC/MS analysis, triclosan was methylated. Consequently, it is possible that the concentration of methyl triclosan attributed to be through metabolic processes may not be accurate. Ethylation of triclosan, rather than methylation, may increase the reliability of the assay for differentiating between this compound and its biological metabolite.

Caffeine is one of the most widely used stimulant drugs and has commonly been used as an anthropogenic marker for wastewater contamination of aquatic environments (143). It was detected in FE and MF wastewater on multiple occasions during the Spring/Summer 2005 and Winter 2006 experiments (Table 4.5-4.10). While membrane UF did not reduce caffeine concentrations in MF wastewater, passage through the GAC unit effectively removed caffeine, with the exception of April 18, 2006. On this date caffeine was detected at low levels only in MCF wastewater, possibly due to a false positive, as this compound was absent in either FE or MF wastewater. Alternatively, caffeine may have been present in FE and MF wastewater on April 18, 2006, but for unknown reasons not properly measured or reported.

4.3 Discussion

Pesticides and pharmaceuticals from agriculture and human medical care have been detected in sewage water, in surface water, and groundwater (2; 140; 144; 145). Based on my research, a variety of herbicides (2,4-D, MCPP/Mecoprop®, MCPA, 2,4-

dichlorophenol, and dicamba/Banvel®), pharmaceutical and personal care products were detected in final treated effluent. These included analgesic/anti-inflammatory drugs (ibuprofen, naproxen, diclofenac, salicylic acid), antiepileptic agents (carbamazepine), cholesteral-lowering agents (gemfibrozil, bezafibrate), anti-bacterial agents (methyl triclosan and triclosan) and caffeine. Some of these xenobiotics were present in the effluent at high concentrations up to 2.3 μg/L, for example, diclofenac and carbamazepine.

Due to the rising concern about environmental issues, the proper operation and control of wastewater treatment plants has become very important. Various conventional physical and chemical treatment processes are being used for removal chemical contaminants from wastewater, including coagulation, polymer and mineral sorbents, reverse osmosis, chemical oxidation, membrane filtration and activated carbon adsorption (146; 147; 65; 148; 149). Studies have shown that the UF membrane process has the potential ability to reduce particles with a molecular weight of greater than 300 Da such as bacteria and protozoa from effluent, as well as the removal of inorganic substances such as heavy metals via electrostatic interactions between the ions and membranes (149). In some studies, membrane nanofiltration has been successfully applied to remove heavy metals with more than 90% efficiency from landfill leachate (150). Another advantage of UF membranes is that it is possible to use them on a large scale effectively and economically compared to activated carbon filtration alone. Ultra-membrane filtered water can be used for industrial or agricultural purposes, for example, watering of golf courses. In spite of its advantages, the major drawback of UF membranes is the low removal efficiency for many non-metallic chemical contaminants. For example, Oota *et al.* (151) found no significant differences in the removal of several main endocrine disrupters after membrane UF treatment. From the data shown in this research, the UF membrane usually removed less than 20% of most chemicals measured, if any reduction of the xenobiotics occurred. Therefore, the reuse of wastewater for indirect purposes will need more advanced treatment than the UF membrane alone if xenobiotic contamination is a concern.

Activated carbon adsorption is a mass transfer process by which a substance is transferred from the liquid phase to the surface of granular activated carbon, and becomes

bound by physical and/or chemical interactions (148). Due to its inherent physical properties, large surface area, microporous structure, high adsorption capacity and surface reactivity (152), it is considered as one of the best available technologies for the reduction of organic compounds from water. Ternes *et al.* (146) investigated the removal of selected pharmaceuticals (bezafibrate, clofibric acid, carbamazepine and diclofenac) in laboratory, pilot scale and in full-scale waterworks and found that pharmaceuticals were almost completely removed by activated carbon treatment with the exception of clofibric acid. Our research showed that the GAC treatment has much higher removal ability of chemicals including pesticides and pharmaceuticals compared to that of the UF membrane alone. For all the chemicals detected, the removal efficiencies by GAC generally reached greater than 70%, and, in some cases the removal was highly efficient and resulted in concentrations below detection limits (e.g. caffeine, ibuprofen, carbamazepine, 2,4-dichlorophenol and triclosan). Despite the high xenobiotic reduction efficiency, the need for frequent replacement of the GAC columns and the cost of GAC may limit its application for the treatment of wastewater on large scale, especially in developing countries.

I believe that use of an UF membrane prior to GAC treatment would increase the longevity and economic feasibility of the GAC units, and potentially allow for a more efficient method of producing safer re-usable water. However, based on my results, a combination of both an UF membrane and GAC treatment is not a perfect solution for the production of high quality water, as GAC treatment did not always completely remove all chemicals, especially when their concentration were low. For example, when salicylic acid levels were low (June 20, 2005), no removal of chemicals was found in MCF wastewater compared to that MF wastewater. The efficiency of the GAC unit was undoubtedly influenced by how long the effluent was in contact with the activated carbon, and therefore by the material, volume, and retention time of the GAC unit. By changing the flow rate and chemistry characteristics of effluent within the GAC unit, the removal efficiency of chemicals could be greatly altered. Thus, further investigations are needed to optimize parameters of both the UF membrane filter and the GAC unit such that large-scale production of re-usable water containing the minimum levels of xenobiotics possible can be produced in the most efficient and economic manner.

However, it should be noted that while chemical analysis of wastewater can help us determine what xenobiotics an organism in the environment may be exposed to, it does not necessarily reflect the impact of these chemicals on the behaviour or physiology of that animal. Mixtures of xenobiotics may act in an additive, synergistic or inhibitive manner on a physiological response that cannot always be predicted using single-toxicant does response experiments (95; 96). In order to determine the possible risks of xenobiotic exposure, a living organism must be used in addition to chemical analysis. Consequently, the following chapters describe the results of a number of such toxicological studies that involved the *in vivo* exposure of goldfish (*Carassius auratus*) to FE, MF or MCF wastewater.

Parameters	FE	MF	MCF
Temperature (°C)	20.7±1.0	19.9±1.2	19.9±1.1
pH	7.18±0.18	7.91±0.15	7.91±0.17
Dissoloved Oxygen (mg/L)	6.17±0.56	6.35±0.50	6.63±0.37
Chlorine (mg/L)	0.01±0.00	0.01±0.00	0.01±0.00
Conductivity (µS/cm)	1026±62	1015±58	1006±92
Salinity (ppt)	0.5±0.00	0.50±0.00	0.50±0.00
Flow Rate (L/min)	1.0±0.1	1.0±0.1	1.0±0.1

FE- final treated effluent; MF- UF membrane effluent; MCF- UF membrane and GAC unit effluent.

Table 4.1 Daily water chemistry characteristics measured in wastewater effluents between April-July 2005 (Spring/Summer 2005 experiment).

Parameters	FE	MF	MCF
Temperature (°C)	16.8±0.8	16.8±0.9	17.1±0.9
pH	7.58±0.15	7.77±0.09	7.78±0.17
Dissoloved Oxygen (mg/L)	5.53±0.68	5.85±0.73	6.17±0.70
Chlorine (mg/L)	0.01±0.01	0.01±0.01	0.01±0.01
Conductivity (µS/cm)	999±146	923±61	919±65
Salinity (ppt)	0.5±0.1	0.4±0.1	0.4±0.1
Flow Rate (L/min)	1.1±0.1	1.0±0.1	1.0±0.1

FE- final treated effluent; MF- UF membrane effluent; MCF- UF membrane and GAC unit effluent.

Table 4.2 Daily water characteristics measured in wastewater effluents between January-April 2006 (Winter 2006 experiment).

Parameters	FE	MF	MCF
Temperature (°C)	21.9±1.5	21.4±1.5	21.33±1.45
pH	7.77±0.19	7.97±0.17	7.95±0.90
Dissoloved Oxygen (mg/L)	6.59±0.49	6.76± 0.51	7.03±0.34
Chlorine (mg/L)	0.02±0.02	0.02±0.01	0.01±0.01
Conductivity (µS/cm)	907±91	1077±106	1063±96
Salinity (ppt)	0.5±0.1	0.5±0.1	0.5±0.1
Flow Rate (L/min)	1.0±0.1	1.0±0.1	0.9±0.1

FE- final treated effluent; MF- UF membrane effluent; MCF- UF membrane and GAC unit effluent.

Table 4.3 Daily water characteristics measured in wastewater effluents between June-August 2006 (Summer 2006 experiment).

Compound	Class	MDL (µg/L)
Bromacil	Herbicide	0.030
2,4-D	Herbicide	0.005
2,4 Dichlorophenol (metabolite)	Herbicide	0.010
Dicamba (Banvel®)	Herbicide	0.005
MCPA	Herbicide	0.005
MCPP (Mecoprop®)	Herbicide	0.005
Triclopyr	Herbicide	0.010
Diazinon	Pesticide	0.005
Carbamazepine	Anti-epileptic/anti-mania agent	0.010
Bezafibrate	hypolipidemic agent	0.100
Gemfibrozil	hypolipidemic agent	0.005
Diclofenac	NSAID/analgesic	0.010
Ibuprofen	NSAID/analgesic	0.005
Naproxen	NSAID/analgesic	0.005
Salicylic Acid	NSAID/analgesic	0.025
Caffeine	Stimulant	0.020
Triclosan	Antibacterial/Antifungal agent	0.010
Methyl Triclosan	Tricolsan metabolite	0.010

Table 4.4 Xenobiotics detected in municipal wastewater and their method detection limits (MDL), as measured by gas chromatography/mass spectrometry.

Pollutants	FE Wastewater				
	April 21 2005	May 1 2005	May 12 2005	June 20 2005	July 19 2005
Pesticides					
Bromacil	nd	nd	nd	nd	nd
2,4-Dichlorophenol	nd	nd	0.445±0.036	0.431±0.006	0.033±0.006
Dicamba	nd	nd	0.016±0.000	0.031±0.001	0.025±0.001
MCPA	0.007±0.002	0.007±0.000	nd	0.029±0.001	nd
Triclopyr	nd	nd	nd	0.031±0.006	nd
Diazinon	nd	nd	nd	0.008±0.000	nd
PPCP compounds					
Bezafibrate	0.200±0.000	nd	nd	nd	0.198±0.010
Caffeine	0.210±0.028	0.016±0.002	0.119±0.002	nd	nd
Methyl Triclosan	nd	nd	nd	0.009±0.001	nd

nd-not detected

Table 4.5 Mean concentration (μg/L ± SEM) of xenobiotics infrequently detected in final effluent (FE wastewater) between April-July 2005 (Spring/Summer 2005 experiment).

Pollutants	MF Wastewater				
	April 21 2005	May 1 2005	May 12 2005	June 20 2005	July 19 2005
Pesticides					
Bromacil	nd	nd	nd	nd	nd
2,4-Dichlorophenol	nd	nd	0.456±0.029	0.456±0.038	0.059±0.038
Dicamba (Banvel)	nd	nd	0.013±0.001	0.036±0.004	0.028±0.004
MCPA	0.006±0.001	0.007±0.000	nd	0.029±0.001	0.007±0.001
Triclopyr	nd	nd	nd	0.035±0.002	nd
Diazinon	nd	nd	nd	nd	nd
PPCPs compounds					
Bezafibrate	0.200±0.00	nd	nd	nd	0.140±0.011
Caffeine	0.200±0.078	0.017±0.001	0.194±0.011	0.027±0.004	nd
Methyl Triclosan	nd	nd	nd	0.007±0.001	nd

nd-not detected

Table 4.6 Mean concentration (μg/L ± SEM) of xenobiotics infrequently detected in membrane ultrafiltration effluent (MF wastewater) between April-July 2005 (Spring/Summer 2005 experiment).

Pollutants	MCF Wastewater			
	May 1 2005	May 12 2005	June 20 2005	July 19 2005
Pesticides				
Bromacil	nd	nd	0.042±0.004	nd
2,4-Dichlorophenol	nd	nd	nd	nd
Dicamba (Banvel)	nd	0.012±0.004	0.022±0.003	0.013±0.003
MCPA	0.006±0.000	nd	0.007±0.000	nd
Triclopyr	nd	nd	nd	nd
Diazinon	nd	nd	nd	nd
PPCP compounds				
Bezafibrate	nd	nd	nd	nd
Caffeine	nd	0.125±0.025	nd	nd
Methyl Triclosan	nd	nd	nd	nd

nd-not detected; *note - the GAC unit was not functioning on April 21, 2005)

Table 4.7 Mean concentration (μg/L ± SEM) of xenobiotics infrequently detected in membrane ultrafiltration and granular activated carbon effluent (MCF wastewater) between May-July 2005 (Spring/Summer 2005 experiment).

Pollutants	FE Wastewater				
	Jan 17 2006	Jan 23 2006	Feb 6 2006	Mar 16 2006	April 18 2006
Pesticides					
2,4-Dichlorophenol	nd	nd	nd	nd	0.027±0.001
Dicamba	nd	nd	nd	nd	0.004±0.001
MCPA	nd	nd	nd	nd	nd
PPCP compounds					
Bezafibrate	nd	nd	nd	nd	0.163±0.016
Caffeine	0.051±0.006	0.088±0.014	0.468±0.089	nd	nd
Methyl Triclosan	nd	0.056±0.001	0.014±0.005	nd	nd

nd-not detected

Table 4.8 Mean concentration (µg/L ± SEM) of xenobiotics infrequently detected in final effluent (FE wastewater) between January-April 2006 (Winter 2006 experiment).

Pollutants	MF Wastewater				
	Jan 17 2006	Jan 23 2006	Feb 6 2006	Mar 16 2006	April 18 2006
Pesticides					
2,4-Dichlorophenol	nd	nd	nd	nd	0.017±0.002
Dicamba	nd	nd	nd	nd	0.005±0.001
MCPA	nd	nd	0.024	nd	nd
PPCP compounds					
Bezafibrate	nd	nd	nd	nd	0.161±0.023
Caffeine	0.051±0.000	0.037±0.004	0.468±0.089	nd	nd
Methyl Triclosan	nd	nd	nd	0.006±0.010	nd

nd-not detected

Table 4.9 Mean concentration (µg/L ± SEM) of xenobiotics infrequently detected in membrane ultrafiltration effluent (MF wastewater) between January-April 2006 (Winter 2006 experiment).

Pollutants	MCF Wastewater				
	Jan 17 2006	Jan 23 2006	Feb 6 2006	Mar 16 2006	April 18 2006
Pesticides					
2,4-Dichlorophenol	nd	nd	nd	nd	nd
Dicamba	nd	nd	nd	nd	nd
MCPA	nd	nd	nd	nd	nd
PPCP compounds					
Bezafibrate	nd	nd	nd	nd	0.057±0.004
Caffeine	nd	nd	nd	0.062±0.012	nd
Methyl Triclosan	nd	nd	nd	nd	nd

nd-not detected

Table 4.10 Mean concentration (µg/L ± SEM) of xenobiotics infrequently detected in membrane ultrafiltration and granular activated carbon effluent (MCF wastewater) between January-April 2006 (Winter 2006 experiment).

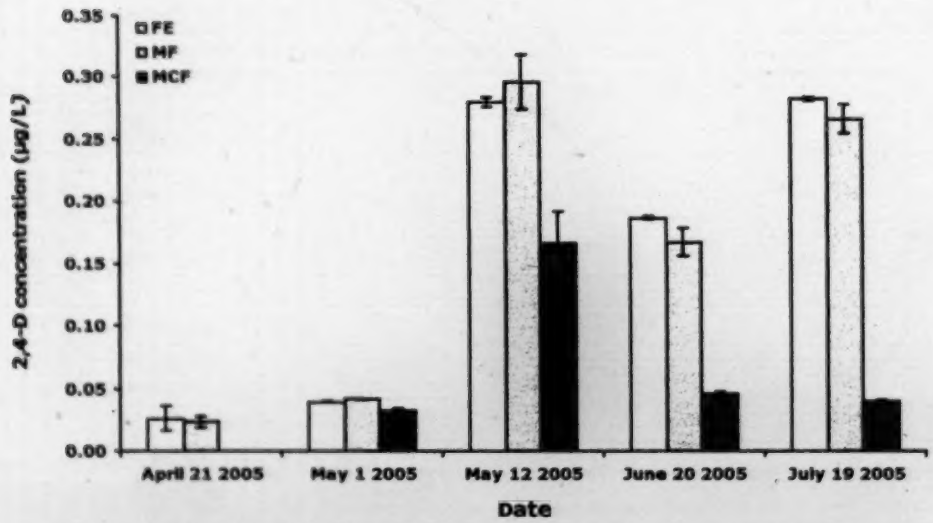

Figure 4.1 Mean concentration (µg/L) ± SEM of the herbicide 2,4-D measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Spring/Summer 2005 experiment. The GAC unit was not functioning on the April 21, 2005 and, as such, this sample date serves as a control. Method detection limit = 0.005 µg/L.

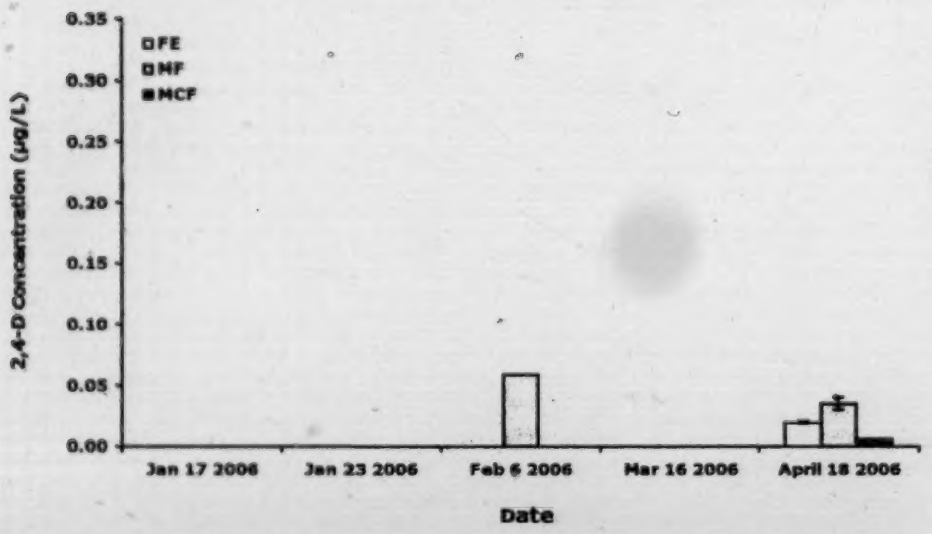

Figure 4.2 Mean concentration (µg/L) ± SEM of the herbicide 2,4-D measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Winter 2006 experiment. Method detection limit = 0.005 µg/L.

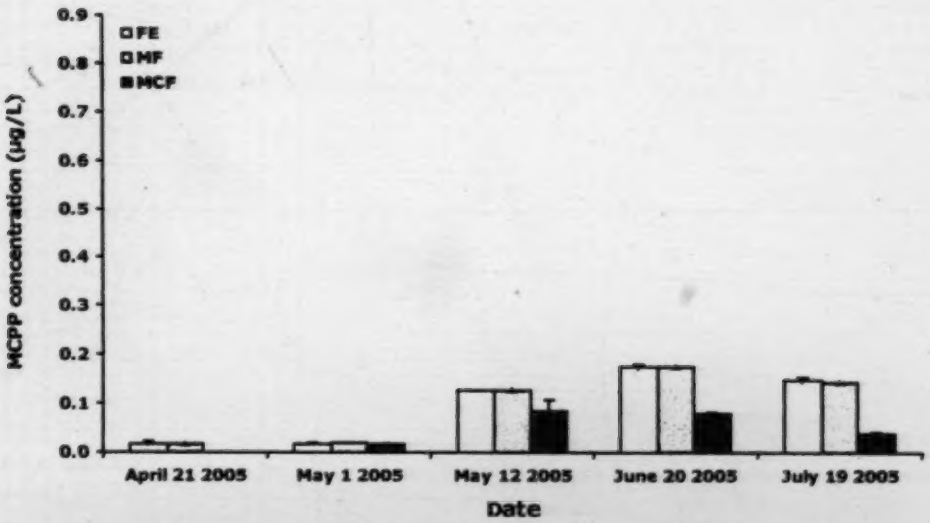

Figure 4.3 Mean concentration (µg/L) ± SEM of the herbicide MCPP measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Spring/Summer 2005 experiment. The GAC unit was not functioning on the April 21, 2005 and, as such, this sample date serves as a control. MDL = 0.005 µg/L.

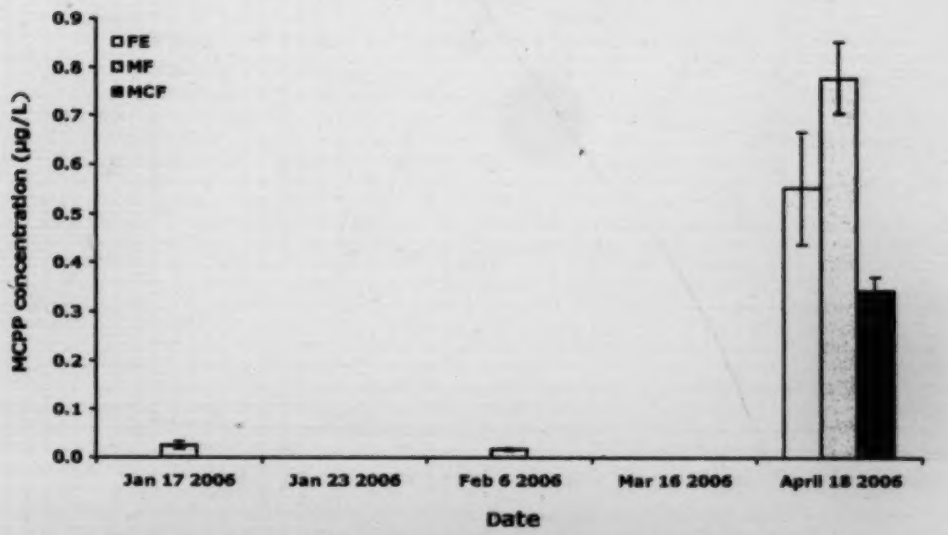

Figure 4.4 Mean concentration (µg/L) ± SEM of the herbicide MCPP measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Winter 2006 experiment. Method detection limit = 0.005 µg/L.

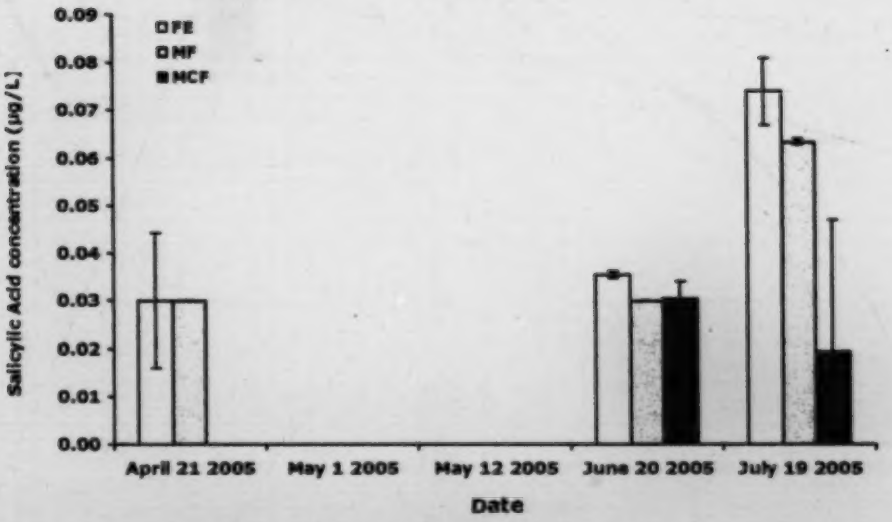

Figure 4.5 Mean concentration (µg/L) ± SEM of the NSAID salicylic acid measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Spring/Summer 2005 experiment. The GAC unit was not functioning on the April 21, 2005 and, as such, this sample date serves as a control. Method detection limit = 0.025 µg/L.

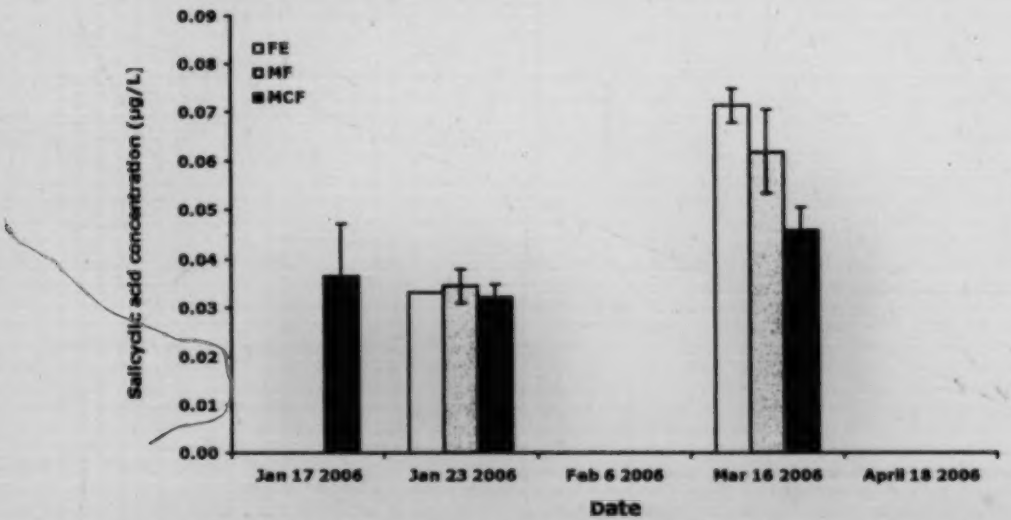

Figure 4.6 Mean concentration (µg/L) ± SEM of the NSAID salicylic acid measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Winter 2006 experiment. Method detection limit = 0.025 µg/L.

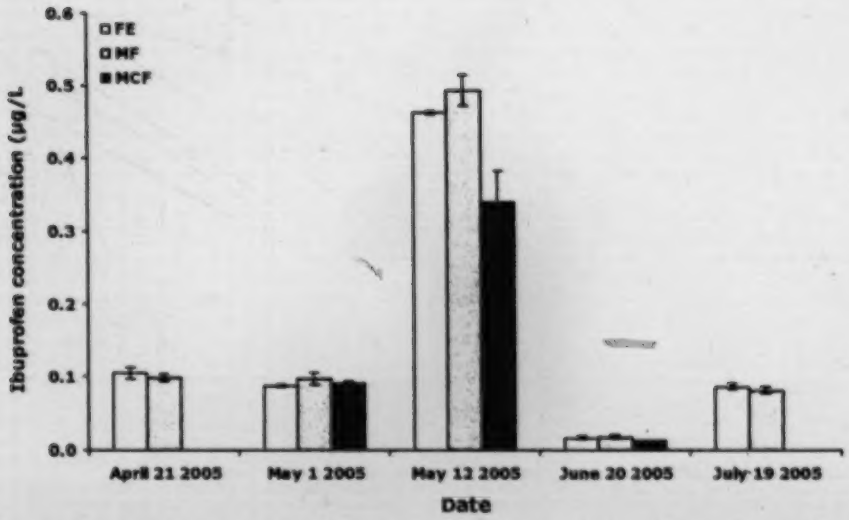

Figure 4.7 Mean concentration (µg/L) ± SEM of the NSAID ibuprofen measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Spring/Summer 2005 experiment. The GAC unit was not functioning on the April 21, 2005 and, as such, this sample date serves as a control. Method detection limit = 0.005 µg/L.

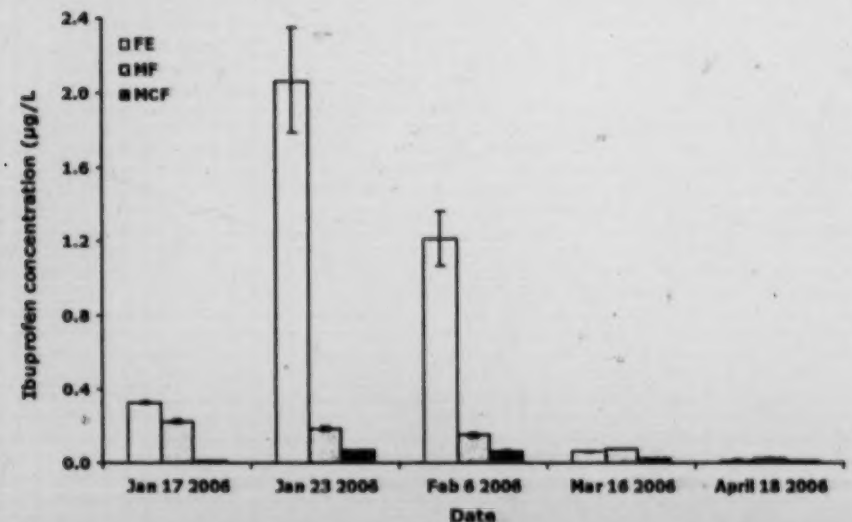

Figure 4.8 Mean concentration (µg/L) ± SEM of the NSAID ibuprofen measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Winter 2006 experiment. Method detection limit = 0.005 µg/L.

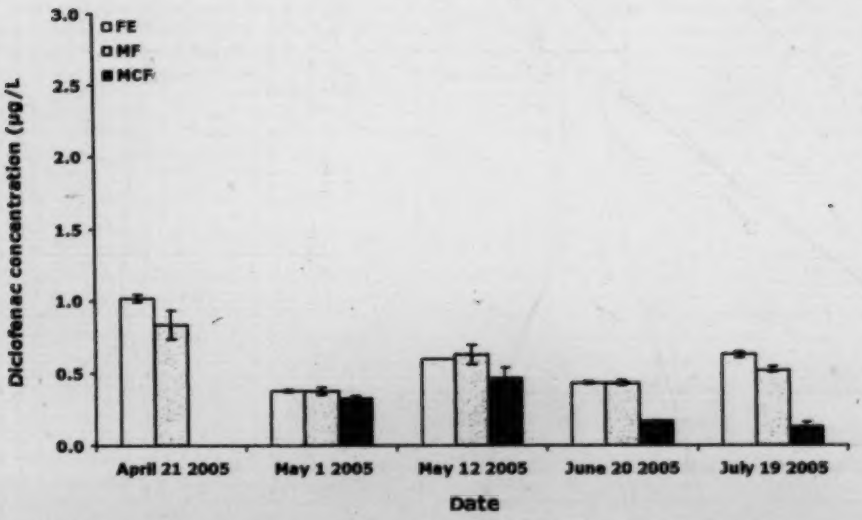

Figure 4.9 Mean concentration (µg/L) ± SEM of the NSAID diclofenac measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Spring/Summer 2005 experiment. The GAC unit was not functioning on the April 21, 2005 and, as such, this sample date serves as a control. Method detection limit = 0.010 µg/L.

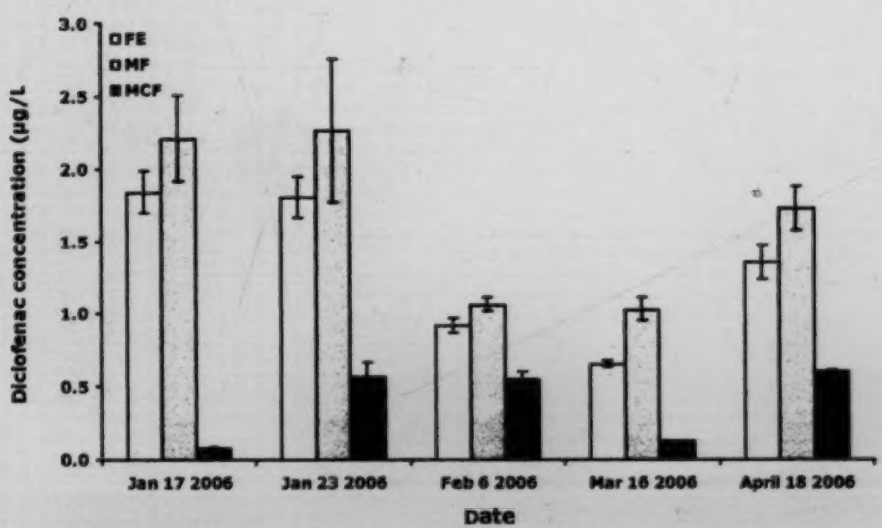

Figure 4.10 Mean concentration (µg/L) ± SEM of the NSAID diclofenac measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Winter 2006 experiment. Method detection limit = 0.010 µg/L.

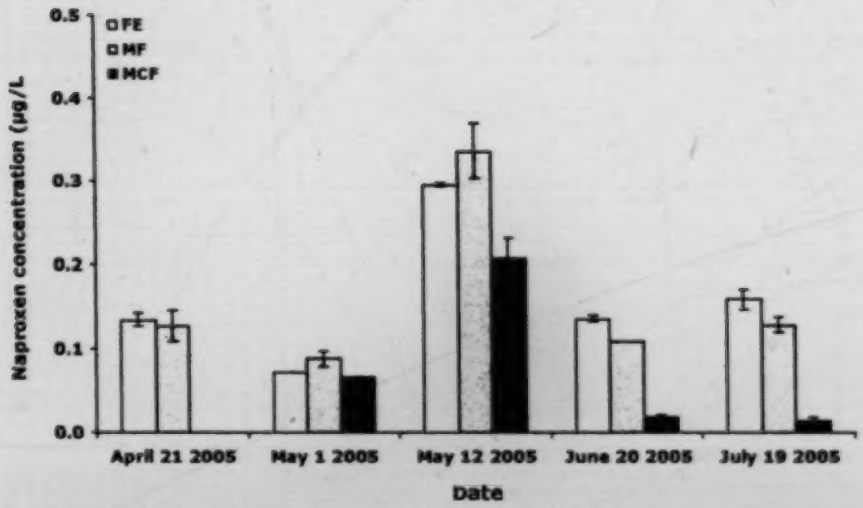

Figure 4.11 Mean concentration (µg/L) ± SEM of the NSAID naproxen measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Spring/Summer 2005 experiment. The GAC unit was not functioning on the April 21, 2005 and, as such, this sample date serves as a control. Method detection limit = 0.005 µg/L.

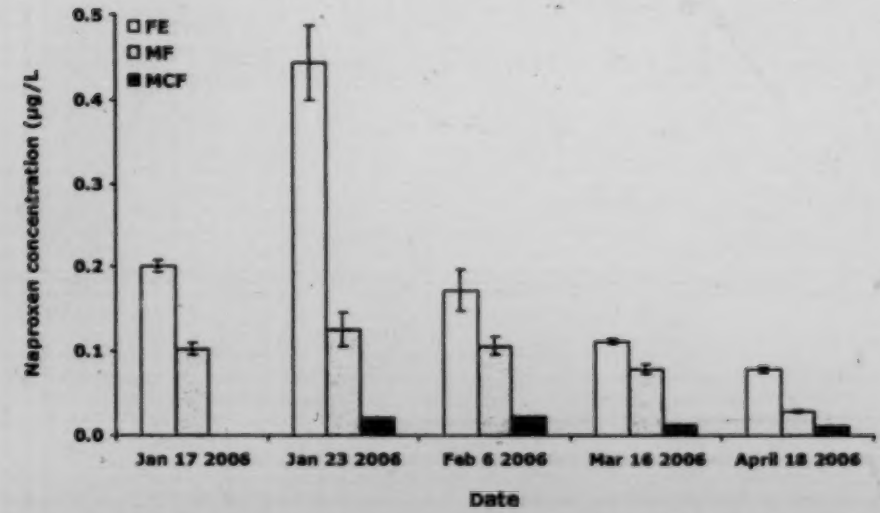

Figure 4.12 Mean concentration (µg/L) ± SEM of the NSAID naproxen measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Winter 2006 experiment. Method detection limit = 0.005 µg/L.

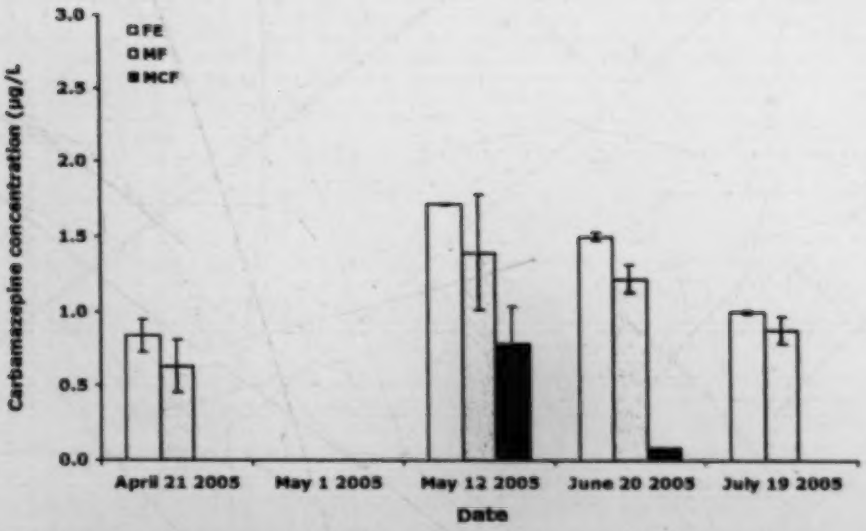

Figure 4.13 Mean concentration (µg/L) ± SEM of the anti-epileptic/anti-mania pharmaceutical carbamazepine measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Spring/Summer 2005 experiment. The GAC unit was not functioning on the April 21, 2005 and, as such, this sample date serves as a control. Method detection limit = 0.010 µg/L.

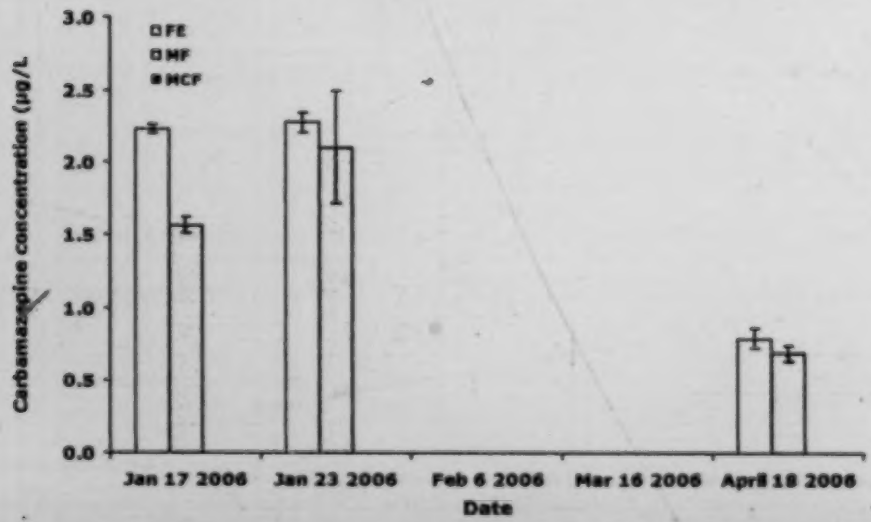

Figure 4.14 Mean concentration (µg/L) ± SEM of the anti-epileptic/anti-mania pharmaceutical carbamazepine measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Winter 2006 experiment. Method detection limit = 0.010 µg/L.

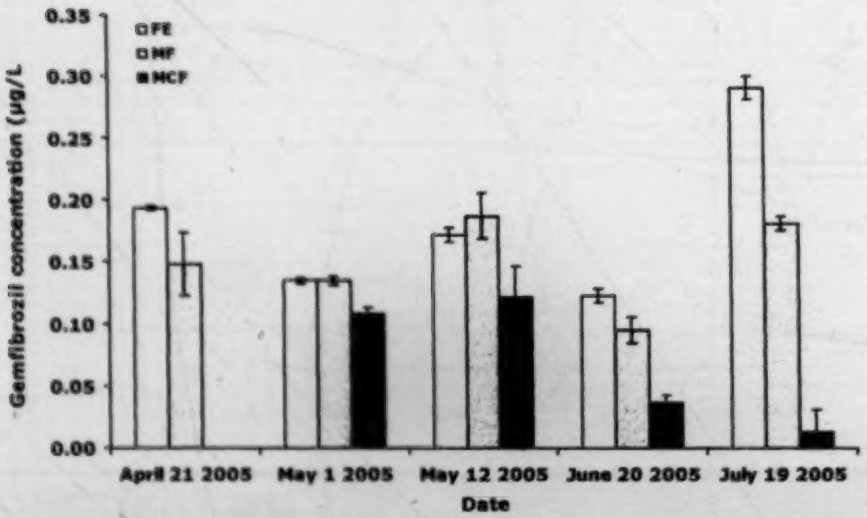

Figure 4.15 Mean concentration (µg/L) ± SEM of the lipid-regulating pharmaceutical gemfibrozil measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Spring/Summer 2005 experiment. The GAC unit was not functioning on the April 21, 2005 and, as such, this sample date serves as a control. Method detection limit = 0.005 µg/L.

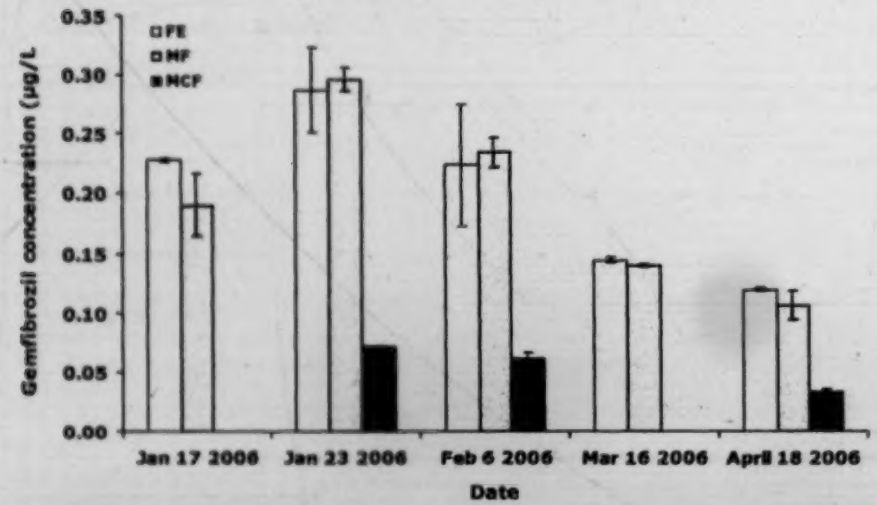

Figure 4.16 Mean concentration (µg/L) ± SEM of the lipid-regulating pharmaceutical gemfibrozil measured in final treated effluent (FE), ultrafiltration membrane effluent (MF), and combined ultrafiltration membrane and granular activated carbon (GAC) filtered effluent (MCF) on various dates during the Winter 2006 experiment. Method detection limit = 0.005 µg/L

CHAPTER FIVE

P450 (CYP1A) AND VITELLOGENIN INDUCTION IN GOLDFISH EXPOSED TO WASTEWATER

5.1 *Introduction*

P450 (CYP1A) induction is a widely accepted biomarker for environmental xenobiotic contamination or chemical exposure in teleost fish, specifically exposure to aryl hydrocarbons (5). Induction of this family of proteins is measured by a number of assays, one of the most common of which is the 7-ethoxyresorufin-O-deelthylase (EROD) assay. In brief, the EROD assay quantitatively measures the activity of P450 (CYP1A) enzymes through the conversion of a substrate, 7-ethoxyresorufin, into a product, resorufin, which can be quantified through fluorescence at appropriate excitation and emission wavelengths. Increased EROD activity is indicative of increased enzyme catalytic activity and, indirectly, an increased amount of previous aromatic hydrocarbon exposure. EROD activity has been shown to be a good bioindicator of exposure to PCBs and PAHs (153; 5) and may be induced in numerous fish species following exposure to municipal wastewater (128; 32).

Vitellogenin (Vtg) induction is another common biomarker of xenobiotic exposure in fish and may be used to indirectly determine whether estrogenic compounds are present in an aquatic ecosystem or wastewater samples to which fish are exposed. Unlike other biomarkers of estrogenic contamination, such as the feminization of male fish reproductive tissue, Vtg induction does not require the fish to be exposed during specific life cycle stages. Furthermore, Vtg induction may be used to determine estrogenic exposure in male fish at virtually any age. Vitellogenin is an egg-yolk precursor protein that is naturally detected in the plasma of reproductively active female teleost fish and is produced in response to endogenous estrogens. Male fish and sexually immature female fish do not normally produce significant amounts of Vtg, but are sensitive to endocrine disrupting compounds such as 17β-estradiol (E2), estrone, 17α-ethinylestradiol (EE2), alkylphenol polyethoxylates (APEs) and PCBs (154; 155). Exposure of fish to these estrogenic compounds may result in a quantifiable induction of

Vtg, as measured in the plasma of adult male or immature fish. Natural or synthetic estrogenic compounds are commonly detected in municipal sewage treatment plant effluents (74; 15; 64; 42; 48) and Vtg induction in male fish exposed to sewage effluent for various periods of time have been reported (23; 129; 32; 130).

As demand for potable water increases and access to suitable water sources diminish, the production of reuse or recycled wastewater obtained through further treatment of municipal final sewage effluent is likely to play a greater role in the water budgets of many regions in the world. As such, the safety of this recycled water on both society and the environment is of utmost importance if we wish to reduce humanity's footprint on the ecosystem.

In this research, I was interested in using goldfish as biosentinels for any potential detrimental effects of wastewater exposure, as well as the effects of reuse water on animal physiology. Reuse water was produced through passage of final sewage effluent (FE wastewater) through an UF membrane (MF wastewater), or UF membrane effluent that was then subsequently filtered using a granular activated carbon (GAC) unit to reduce xenobiotic levels in the reuse water (termed MCF wastewater).

5.2 *Results*

Cytochrome P450 (CYP1A) enzymatic activity was determined in liver microsomes isolated from goldfish exposed to differently treated municipal wastewater, using the EROD assay. In addition to a control group that were killed prior to wastewater exposure, fish were sampled following exposure to FE, MF or MCF wastewater for 7, 21, 60 and 90 days, either in a Spring/Summer experiment (April through July, 2005) or in a Winter experiment (January through April, 2006). EROD activity differed significantly between the Spring/Summer 2005 and Winter 2006 experiments, and consequently the results for each experiment were analysed separately.

Vitellogenin (Vtg) induction, a common biomarker of estrogenic compound exposure, was analysed in fish exposed to different municipal wastewater treatments between January and April 2006 (Winter 2006 experiment). Plasma samples were isolated from male fish sampled at the same time points listed for the P450 (CYP1A)

induction studies, flash frozen in liquid nitrogen, and quantified using pre-coated carp Vtg ELISA plates (Biosense Laboratories). Plasma samples from male fish exposed to the municipal wastewater treatments during the Spring/Summer 2005 experiment were not analysed due to insufficient sample size. During the Winter 2006 experiment both the length of time the fish were exposed to the wastewater, the wastewater treatment itself and the interaction between the two were found to be highly significant ($P<0.001$).

5.2.1 P450 (CYP1A) induction in goldfish exposed to different wastewaters (Spring/Summer 2005 experiment)

The EROD activity in fish microsomes from the Spring/Summer 2005 experiment ranged from 2.7 pM resorufin/μg protein/min in Day 90 MCF fish to 7.4 pM resorufin/μg protein/min in Day 90 FE fish; approximately a 2.7 fold induction (Fig. 5.1). The sex of the fish did not significantly affect EROD activity ($P>0.05$), indicating that during late spring and summer the gender of the fish does not impact the catalytic activity of P450 (CYP1A) enzymes. In addition, no significant cumulative effect of the length of wastewater exposure was observed ($P>0.05$). Wastewater treatment was found to significantly affect EROD activity ($P<0.05$); however, only FE exposed fish were found to have significantly increased EROD activity over MCF exposed fish ($P<0.05$). When comparing wastewater-exposed fish to control fish (one-way ANOVA, significance indicated by asterisks in Fig. 5.1), only Day 90 FE fish had significantly induced EROD activity ($P<0.05$).

5.2.2 P450 (CYP1A) induction in goldfish exposed to different wastewaters (Winter 2006 experiment)

The mean EROD activity of liver microsomes from fish exposed to municipal wastewater during the winter of 2006 did not differ significantly between male and female goldfish ($P>0.05$). Time (days of exposure), wastewater treatment and the interaction between the two all were highly significant (multifactoral ANOVA, $P<0.001$). EROD activity ranged from an average of 3.6 pM resorufin/μg protein/min (Day 21 MCF wastewater exposed fish) to 22.4 pM resorufin/μg protein/min (Day 90 MF wastewater exposed fish); approximately a 6-fold difference (Fig. 5.2). In general, when examining the effect of wastewater exposure for each time point, MF wastewater exposed fish had

significantly higher average EROD activity compared to MCF exposed fish at each time point except Day 60 ($P<0.05$; statistical significance indicated by differing lower case letters in Fig. 5.2). The average EROD activity of fish exposed to FE wastewater was not statistically significant compared to that of fish exposed to MCF wastewater, or that of fish exposed to MF wastewater ($P>0.05$). This was likely due to the lower mean EROD activity of FE wastewater exposed fish and the increased within-group variability. While statistical significance may not have been met, fish exposed to FE wastewater had mean EROD activities as much as 3-fold greater than fish exposed to MCF wastewater at the same time point, indicating that exposure of these fish to FE wastewater induced an increase in EROD activity that may be biologically significant.

When examining temporal effects of exposure for each wastewater treatment group, the average EROD activity of both fish exposed to FE wastewater and MF wastewater did not differ over time ($P>0.05$; statistical significance indicated by differing capital letters on Fig. 5.2). For fish exposed to MCF wastewater, EROD activity was significantly higher on Day 60 compared to other time points ($P<0.05$), but insignificant among other time points.

During the normal course of the experiment, the activated carbon and glass wool within the GAC unit was manually replaced every four to five weeks. Frequently within the last week prior to replacement, a reduced flow rate from the GAC unit was observed compared to previous weeks', as manifested by a reduced degree of overflow in the MCF effluent head tank. Approximately one week prior to the Day 60 sampling time point (March 16, 2006) a reduced flow rate was observed from the GAC unit, and the activated carbon was replaced five days prior to the sampling point. On Day 60, the activated carbon filter was functioning with a similar degree of xenobiotic removal efficiency as in previous sampling time points (see Chapter 4). However, fish exposed to MCF wastewater sampled at this time point exhibited significantly higher levels of EROD activity than previous time points, indicating the fish had been exposed to aryl hydrocarbon receptor agonists. This exposure likely occurred because of a GAC filter malfunction that was undetected due to the periodic nature of our xenobiotic analysis in the wastewater effluents. The increase in EROD activity in fish exposed to MCF wastewater at Day 60 illustrates the strength and sensitivity of the EROD assay in

detecting temporal changes in water quality that may not necessarily be detected through periodic chemical analysis of wastewater alone. The Day 60 induction of EROD activity in fish exposed to MCF wastewater exposed fish did not exert any long-term cumulative effects on CYP1A induction: by Day 90, EROD activity in fish exposed to MCF wastewater was reduced and did not differ from that of fish exposed to MCF wastewater on Days 7, 21 or control fish (Fig. 5.2). Interestingly, a study by Gagnon and Holdway (214) also showed an increase in hepatic EROD activity in fish following exposure to aryl hydrocarbon receptor agonists (crude oil) that was found to persist for 4-6 days after transfer to clean water. The authors also found that subsequent depuration of exposed fish in clean water for 29 days resulted in a return of EROD activity to that of unexposed control fish.

The EROD activity in fish exposed to wastewater was also compared to that of control fish using a one-way ANOVA (Dunnett's multiple comparisons test; statistical significance indicated by asterisks in Fig. 5.2). Fish exposed to MF wastewater had significantly higher EROD activity compared to control fish on Day 60 and Day 90 ($P<0.05$ and $P<0.01$, respectively). Fish exposed to MCF wastewater on Day 60 post-exposure, as well as fish from the Day 90 FE exposure group were found to have EROD activity significantly higher than control fish ($P<0.01$; $P<0.05$, respectively).

5.2.3 Induction of vitellogenin in male goldfish exposed to different wastewaters (Winter 2006 experiment)

When analyzing differences in plasma Vtg concentration between male fish exposed to FE, MF or MCF wastewater, no differences were observed between treatment groups at Day 7 post exposure (statistical significance indicated by differing lower case letters; Fig. 5.3). At Day 21 post exposure, both fish exposed to FE wastewater and those exposed to MF wastewater had significantly higher Vtg levels in the plasma compared to MCF wastewater exposed fish ($P<0.001$), but not between each other ($P>0.05$). At Day 60, even though the average Vtg concentration in fish exposed to MF wastewater had dropped from a peak of 17.6 μg/ml blood at Day 21 to 8.3 μg/ml blood, the same trend of statistical significance seen at Day 21 was observed ($P<0.05$). By Day 90, however, only fish exposed to FE wastewater maintained a significantly higher level of plasma Vtg compared to those exposed to MCF wastewater ($P<0.01$). Fish exposed to FE wastewater

also had significantly greater blood Vtg concentration than fish exposed to MF wastewater ($P<0.001$). Fish exposed to MCF and MF wastewater for ninety days did not differ statistically in terms of Vtg levels ($P>0.05$).

When the data were analysed for trends within a wastewater treatment group over time (statistical significance indicated by differing upper case letters; Fig. 5.3), FE wastewater exposed fish were found to have an initial low level of Vtg induction at Day 7, followed by a sustained high level of Vtg in the blood at Day 21, 60, and 90 (Fig. 5.3). The Vtg concentration at Day 7 was not significantly different from that in fish exposed to FE wastewater for 21 days, even though the average Vtg concentration in the blood rose from 3.3 µg/ml to 17.8 µg/ml blood at Day 21 ($P = 0.065$). This lack of statistical significance was likely due to the large degree of variability observed within the Day 21 group. Fish exposed to FE wastewater for 60 and 90 days had blood Vtg concentration significantly higher than that of Day 7 FE fish ($P<0.05$), but not from Day 21 Vtg levels or from each other ($P>0.05$). For fish exposed to MF wastewater, low levels of Vtg were found in early exposure fish (Day 7), but levels peaked in fish exposed to MF wastewater for 21 days, following which subsequently lower and lower levels were observed for the last two time points (Day 60 and Day 90). Fish exposed to MF wastewater for 7 days had Vtg levels that were not statistically different from any of the following within-group time points. Day 21 MF wastewater exposed fish as well as Day 60 MF fish did have significantly higher Vtg levels than Day 90 MF wastewater exposed fish ($P<0.001$ and $P<0.05$, respectively), but were not significant from each other ($P>0.05$). Regarding MCF wastewater exposed fish, Vtg was not induced at any time point, and no time points differed significantly from each other ($P>0.05$).

When a one-way ANOVA was conducted comparing each experimental group against control male fish (statistical significance indicated by asterisks in Fig. 5.3), fish exposed to FE wastewater were found to have significantly higher Vtg concentrations in their blood than control fish at all time points ($P<0.05$ for Day 7 and $P<0.01$ for each Day 21, 60 and 90). MF wastewater exposed fish were only found to be statistically significant from control fish on Days 21 and 60 ($P<0.01$). Fish exposed to MCF wastewater did not differ from control fish at any time point ($P>0.05$).

5.3 Discussion

In this study, I demonstrated that exposure of goldfish to FE or MF wastewater resulted in an increase in P450 (CYP1A) catalytic activity, indicating aryl hydrocarbon contamination in the wastewater. To the best of my knowledge, this is the first study to address the effects of reuse water exposure on EROD activity in a fish species. Previous studies have also shown that municipal wastewater can induce an increase in P450 (CYP1A) catalytic activity in fish species, as measured by an enhanced EROD activity. For example, diluted (30%) municipal wastewater effluent induced a three-fold increase in EROD activity in rainbow trout (*Oncorhynchus mykiss*) exposed for 27 days, when compared to clean water cohorts (128). Japanese medaka (*Oryzias latipes*) exposed to Beijing sewage treatment plant effluent had a significantly increased EROD activity following exposure to 10%, 20%, 40% and 50% sewage effluent [three to four fold increases over baseline levels; (32)]. In these studies, exposure of fish to municipal wastewater resulted in a similar maximum fold increase in EROD activity as that found in my experiments (<10 fold), indicating that municipal sewage effluent may be a weak EROD inducer.

The low degree of P450 (CYP1A) induction is not surprising when one considers that municipal sewage wastewater should not contain the high levels of cytochrome P450 inducers, such as is found in oil sands sediment (156). In fact, some pharmaceutical products have been shown to inhibit chemically-induced increases in EROD activity *in vitro* (113; 108), albeit at much higher xenobiotic concentrations than were detected in final effluent or reuse water in this study. It is difficult to predict the exact nature or concentrations of CYP1A-inducing chemicals present in Edmonton sewage wastewater or reuse water, as chemical analysis of wastewater did not include screening for volatile or extractable priority pollutants such as PAHs and PCBs [priority status determined through the Canadian Environmental Protection Act, 1999 (71)].

On a number of occasions, variability between individual fish resulted in a lack of statistical significance compared to clean water controls or MCF wastewater exposed fish. However, upon examination of the mean EROD activity of fish exposed to these wastewater conditions, it is clear that exposure to these wastewater effluents resulted in a biologically significant induction of the P450 (CYP1A) enzymatic activity. For example,

during the Winter 2006 experiment the EROD activity of fish exposed to FE wastewater for 7 days did not differ statistically from that of fish exposed to MCF wastewater, even though fish exposed to FE wastewater had 3.1-fold higher mean EROD activity levels than fish exposed to MCF wastewater. On the other hand, MF wastewater exposed fish, which had a similar fold increase in EROD activity levels over that of fish exposed to MCF wastewater (3.6-fold), did differ statistically from MCF exposed fish. Chemical analysis of both FE and MF wastewater indicated similar concentrations of xenobiotics in both these wastewaters.

When comparing P450 (CYP1A) induction in the Spring/Summer 2005 and Winter 2006 experiments, I found that while the overall trends in EROD activity of goldfish exposed to the different wastewater treatments were similar between the two experiments, the mean EROD activity of fish from the Winter 2006 experiment was consistently higher than that of fish from the Spring/Summer 2005 experiment. This relative increase in EROD activity occurred both in terms of wastewater-induced and baseline control values. Furthermore, while the EROD activity in the Spring/Summer 2005 experiment varied by as much as three fold between treatment groups, EROD activity in the Winter 2006 experiment varied by as much as six fold. It is important to note that during the Spring/Summer 2005 experiment liver tissues were frozen using dry ice (-79°C), whereas liquid nitrogen (-196°C) was available to flash-freeze the tissue samples in the Winter 2006 experiment. Because the catalytic activity of the EROD assay changes relative to the amount of functioning P450 (CYP1A) enzymes, and these enzymes are susceptible to degradation by proteases, maximum enzymatic activity should be preserved if tissue was flash frozen because protease enzymes have a reduced amount of time to degrade the P450 (CYP1A) enzymes. Indeed, the EROD activity in control fish or fish exposed to the different wastewater treatments during the Spring/Summer 2005 experiment (liver tissue frozen on dry ice) differed significantly from that of fish exposed during the Winter 2006 experiment (liver tissue flash frozen with liquid nitrogen; $P<0.05$)

Alternatively, in the Winter 2006 experiment goldfish may have been exposed to higher level of aryl hydrocarbons than in the Spring/Summer 2005 experiment. Higher concentrations of a number of different xenobiotics were indeed recorded during the

Winter 2006 experiment compared to the previous experiment. However, it should be noted that the majority of xenobiotics detected in the municipal wastewater in this study are not known to be P450 (CYP1A)-inducing compounds. The compound(s) that caused the induction of EROD activity in wastewater exposed remain to be identified.

A variety of factors can potentially influence P450 (CYP1A) induction in teleosts. For example, increasing water temperature was related to increased EROD activity in female carp (24). It is unlikely that water temperature influenced the overall increase in EROD activity observed in the Winter 2006 experiment since lower mean water temperatures were recorded during this experiment than that found for the Spring/Summer 2005 experiment. Interestingly, during both experiments, the gender of the fish did not significantly impact average EROD activity. This is in contrast to other studies, which found that fish gender and sexual maturity could impact EROD activity in fish, such that female fish from pulp and paper mill effluent contaminated sites displayed significantly reduced levels of EROD activity compared to males or juveniles from the same site (157). This female-specific suppression of EROD activity was not observed in goldfish exposed to FE or MF wastewater.

In short, numerous studies have shown that the EROD assay is a common biomarker for aryl hydrocarbon pollution in environments impacted by chemical pollutants (5). The results of this study confirm that EROD activity may be used to detect the presence of cytochrome P450 inducing compounds in municipal final effluent wastewater and reuse water produced through membrane UF.

Estrogenic compounds are some of the most widely studied xenobiotics. These endocrine disruptors are commonly found in municipal wastewater (74; 100; 64; 42; 48; 158; 59; 75) due to their use as contraceptive or hormone therapy drugs, as well as endogenous secretion. I found that exposure of male goldfish to both MF reuse wastewater and FE wastewater resulted in significant estrogenic effects. However, the pattern of Vtg induction differed between the two wastewater treatments, particularly at later exposure time points. Activated carbon filtration effectively removed estrogenic contamination of municipal wastewater, as detected though a lack of Vtg in MCF wastewater exposed male fish. Vitellogenin induction has been shown by a number of researchers in male teleost fish exposed to municipal wastewater, either directly in a

laboratory setting or in fish exposed downstream of sewage treatment plants (112; 23; 25; 129; 32; 131). Male crucian carp (*Carassius carassius*) exposed to 25%, 50% or 100% treated sewage effluent for 28 days had significantly higher blood Vtg concentration compared to control fish (129). The authors of this study also found reproductive abnormalities such as a lack of spermatogenesis and the presence of oocytes in the testes of some males exposed to 100% effluent. Long-term wastewater exposure may also result in reproductive effects in fish. Juvenile roach (*Rutilus rutilus*) exposed for 150 days to diluted sewage treatment plant effluent had a dose dependant Vtg induction and feminization of reproductive ducts, the latter of which persisted following a 150 day 'clean' water depuration (23).

We found that Vtg was induced in both male fish exposed to FE and MF wastewater. However, at some time points, such as Day 21 post exposure, no statistical significance existed between the plasma Vtg of male fish exposed to FE wastewater compared to those exposed to MCF wastewater. This was likely due to the high variability of plasma Vtg concentration in male FE wastewater fish. Regardless, the mere presence of this female-specific protein in the blood of male fish demonstrates the biological significance of estrogenic compounds in FE wastewater at this time point.

While membrane UF of wastewater did not eliminate estrogenic compounds, as illustrated by a biologically significant induction of Vtg in fish exposed to MF wastewater as early as Day 7 post-exposure, and a statistically significant plasma concentration of Vtg at Day 21 and 60 sampling time points, the pattern of Vtg induction was variable between fish exposed to FE and MF wastewater. Male goldfish exposed to FE wastewater experienced a stable concentration of Vtg in the plasma from Day 21 through 90 post-exposure, while those exposed to MF wastewater had a peak Vtg concentration at Day 21 post exposure that had halved by Day 60 and was eliminated by Day 90. A possible reason for the reduction of Vtg in the plasma of male MF wastewater exposed fish at Day 60 (March 18, 2006) and Day 90 (April 17, 2006) is that these two time points correspond with the advent of spring in Edmonton, AB. During this time, snowmelt into the sewer system of the city could potentially result in an increase in particulate matter present in the final effluent. Significant amounts of estrogens may bind to fine particles (159), and research has indicated that estrogens adsorb to particulate

matter found in sewage effluent (160). If a higher fraction of estrogens bound to the particulate matter during the spring runoff, membrane UF would result in 100% removal of these solids, potentially resulting in a lower bioavailability of estrogens in MF wastewater. Fish exposed to MF wastewater would then have lower Vtg concentrations compared to FE wastewater fish by the later sampling time points, as they would have been able to clear much of the previous estrogen-induced Vtg. In support of this hypothesis, are results of another study (23) that found Vtg induction in male fish exposed to sewage effluent was significantly reduced following subsequent 'clean water' depuration.

While estrogenic compounds may sorb to particulate matter or exist in conjugated forms in wastewater, these endocrine disrupting compounds can be released as free estrogens through deconjugation by microbial enzymes. Consequently, though the bioavailability of estrogens may have been greatly reduced in fish exposed to MF wastewater, fish in the unfiltered FE wastewater (which contained a much higher fecal coliform level than MF wastewater; data not shown) could have been exposed to free estrogens at levels sufficient to maintain sustained plasma Vtg concentration until Day 90. However, it is unknown whether the bioavailabilities of estrogenic compounds differed in the different wastewater conditions with the onset of spring or at any other sampling time point because the concentration of the various estrogens were not measured in this study.

Neither biologically significant nor statistically significant Vtg induction occurred in goldfish exposed to MCF wastewater at any time point in the experiment, indicating that activated carbon filtration effectively removes estrogenic compound contamination from municipal wastewater. If the GAC unit malfunctioned and failed to remove xenobiotics from the MCF wastewater in the week prior to the Day 60 sampling time point, as suggested by the EROD assay data, this failure was not reflected by a Vtg induction in male fish. Maximal Vtg levels were observed in fish exposed to FE or MF wastewater treatments following twenty-one days post exposure. This suggests that constant exposure to estrogenic compounds for as little as seven to twenty-one days is required for significant induction of Vtg.

Though chemical analysis of wastewater samples did not include screening for estrogenic compounds, previous studies have examined the level of estrogenic organic contaminants in Canadian wastewater treatment plants (64; 158; 59; 75). A preliminary survey done by the Government of Alberta in December of 2002 found concentrations of the estrogenic compounds E2 and estrone of 2.08 ng/L and 34.06 ng/L, respectively, in a grab sample of final effluent wastewater at Gold Bar Wastewater Treatment Plant (59). Furthermore, flow-proportional 24 hr composite samples also from Gold Bar during April of 2005 indicated approximately 20 ng/L of total estrogenic equivalent activity in final effluent wastewater (75). Even though this value represents a reduction of greater than 60% of influent estrogenic activity (75), estrogenic effects have been observed in fish at EE2 concentrations as low as 0.32 ng/L (111) and these compounds may potentially impact the Darwinian "fitness" of aquatic species (120).

Municipal sewage effluent and reuse water can contain thousands of xenobiotics such as PPCP compounds (1; 42; 2; 16; 3) and these chemicals may be found in the aquatic environment (1; 161; 10; 2; 140; 162; 66; 59; 13; 3; 60) or sources of drinking water (12; 10; 2; 13). Due to the constant exposure of fish to chemicals in their aquatic environment, water-borne pollutants are likely to first impact these animals prior to affecting terrestrial organisms such as humans. Consequently, it is of great interest to ensure that the potential harmful effects of xenobiotics on organisms in the environment or, potentially, humans are minimized as much as possible.

As shown by the above data, reuse water produced through membrane UF did not consistently remove aryl hydrocarbon receptor agonists or estrogenic compounds, whereas recycled water produced through activated carbon filtration largely prevented the induction of either P450 (CYP1A) enzymatic activity or the estrogen-sensitive biomarker Vtg in exposed goldfish. However, it is important to note that to best evaluate the acute and chronic impacts of these wastewater products on living organisms, these bioassays should be used in conjunction with a suite of other biomarkers. Furthermore, while fish have the potential to serve as biosentinels for pollutants in the aquatic environment, great care must be taken when extrapolating the physiological effects of xenobiotics in goldfish to other fish species or to human populations.

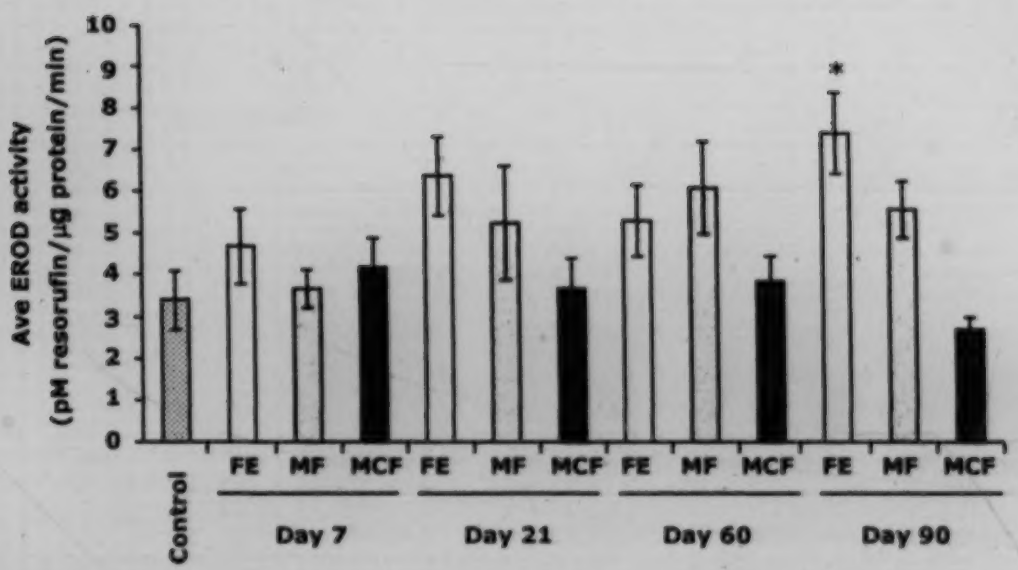

Figure 5.1 Mean (± SEM) 7-ethoxyresorufin 0-deethylase (EROD) activity for liver microsomes isolated from goldfish exposed to differently treated municipal wastewater for up to 90 days between April 2005 and July 2005. Sample size is 10 for all groups except: control (n = 8), Day 90 FE (n = 11) and Day 90 MF (n = 9). Asterisk indicates statistical significance in one-way ANOVA compared to control ($P<0.05$). Factoral ANOVA indicated no significant effect of fish sex or length of exposure, but a significant effect of treatment, with FE being significantly different only from MCF (see results for more details).

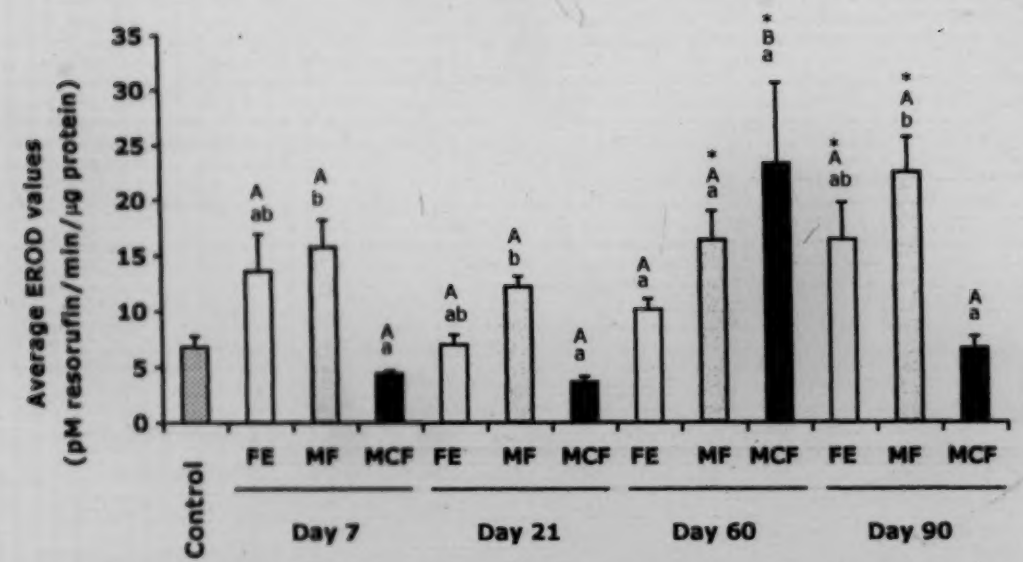

Figure 5.2 Mean (± SEM) 7-ethoxyresorufin 0-deethylase (EROD) activity for liver microsomes isolated from goldfish exposed to differently treated municipal wastewater for up to 90 days between January 2006 and April 2006 (Winter 2006). Sample size is 10 for all groups except: Day 60 MCF (n = 9) and Day 90 MF (n = 9). Asterisks indicates statistical significance in one way ANOVA compared to control ($P<0.05$). Data was analysed using a factoral ANOVA both between treatment groups at each time point (statistical significance indicated with differing lower case letters) and within treatment groups over time (statistical significance indicated with differing upper case letters). No statistical difference was observed between male and female fish.

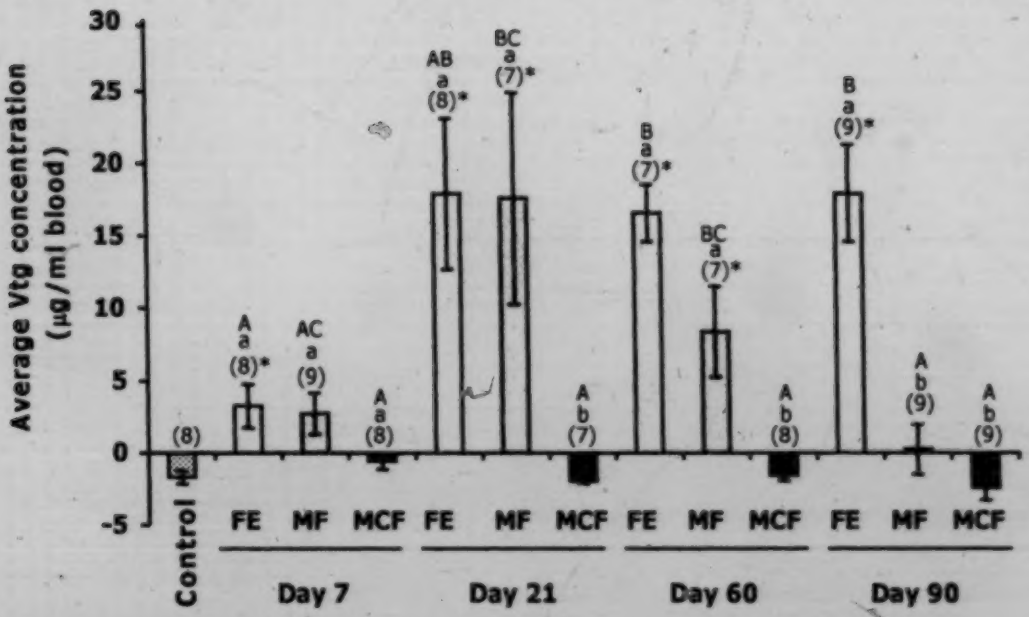

Figure 5.3 Mean (± SEM) plasma vitellogenin (Vtg) concentration of male goldfish exposed to differently treated municipal wastewater for up to 90 days between January 2006 and April 2006 (Winter 2006). Sample size is as indicated. Asterisks indicate statistical significance in one way ANOVA compared to control ($P<0.05$). Data was analysed using a factoral ANOVA both between treatment groups at each time point (statistical significance indicated by differing lower case letters) and within treatment groups over time (statistical significance indicated by differing upper case letters). Assay working range is 0.98-125 ng Vtg/mL and the inter-assay coefficients of variation are between 2.6 and 24% within the working range (Biosense laboratories).

CHAPTER SIX

IMMUNOLOGICAL RESPONSE OF FISH EXPOSED TO DIFFERENT WASTEWATER TREATMENTS

6.1 *Introduction*

The immune system of all organisms, including teleosts, is essential in the defense against invading microorganisms, viruses, and parasites, as well as in the maintenance of internal homeostasis. The first types of cells to encounter antigens are monocytes/macrophages and granulocytes such as neutrophils, followed by lymphocytes. These leukocytes may then undergo activation and proliferation, an essential step if an effective immune response against invading pathogens is to occur.

The activation and/or proliferation of immune cells may be measured using a number of different assays (17). For example, changes in the relative numbers or proportions of leukocytes can be detected using monoclonal antibodies specific to different subpopulations. The relative activity of immune cells may be determined through *in vitro* stimulation with mitogens or antigens and subsequent measurement of mitochondrial enzyme activity (WST-1 reaction). Alternatively, mitochondrial enzyme activity may also be used to indirectly measure proliferation. Proliferation of leukocytes may be measured through ^{3}H-thymidine incorporation or BrdU incorporation into cellular DNA. Interpretation of results that utilize ^{3}H-thymidine must be taken with caution, however, due to the potential for this radioactive tracer to induce cell cycle arrest and apoptosis (163).

Physiological effects of xenobiotic exposure may manifest itself as changes in the relative expression of genetic markers. These genetic markers may be associated with energy metabolism (164-166), the stress response (164; 167; 47), excretion of toxicants (164; 167; 168; 47), growth and development (165; 169), reproduction (168; 165), and the immune response (31; 167). For example, Gravel and Vijayan (47) found that exposure of rainbow trout fry (*O. mykiss*) to environmentally relevant concentrations of ibuprofen (1 μg/L) resulted in a significant induction of the heat shock protein 70 (hsp70), a molecule that aids in protection against stressor-mediated proteotoxicity.

When fish were exposed to higher concentrations of either ibuprofen or another NSAID, salicylate, and then subjected to heat shock, the authors found that only salicylate pre-exposure induced an increase in hsp70 mRNA levels, but both pharmaceuticals caused a reduced or delayed expression of this important protein (47).

The use of gene expression as a biomarker for pollutant exposure is advantageous as only a small amount of tissue is required to isolate sufficient quantities of mRNA, and the expression levels of a large number of different genes may be accurately and quantitatively assessed using real-time PCR from a single sample. Because I wanted to examine the effects of xenobiotic exposure on this most basic level of biology, we chose to examine the expression of a number of immunologically relevant genes (Toll-like receptor 22, tumor necrosis factor α, macrophage colony stimulating factor receptor-1, and granulin) in goldfish following exposure to different wastewater treatments.

Toll-like receptors (TLRs) are evolutionarily ancient, phylogenetically conserved pattern recognition proteins that are major components of innate and acquired immunity (170). They function by recognizing non-self pathogen-associated molecular patterns (PAMPs) present on the cell surface of bacteria and other microorganisms. Binding of PAMPs to TLRs on leukocytes initiate intracellular signaling cascades that ultimately result in the synthesis and secretion of pro-inflammatory cytokines and chemokines. Toll-like receptors have been identified in a number of fish species, including goldfish (171).

Sensitive control of the immune system occurs though the secretion of various molecules such as chemokines and cytokines from activated leukocytes, epithelial and endothelial cells. These compounds regulate proliferation, immune response pathways, inflammation, or suppress immune responses once the pathogen threat has been eliminated. Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine secreted by immune cells such as macrophages. This cytokine is involved in inducing a number of immunological effects in fish including enhancing leukocyte phagocytosis (172), priming of the respiratory burst response (173), cellular migration (172; 173), as well as upregulation of the expression of other pro-inflammatory cytokines such as IL-1β. Studies have found that fish leukocytes will increase transcription of TNFα following stimulation by antigens such as lipopolysaccharide (LPS), the protein kinase C activator

phorbol 12-myristate 13-acetate [PMA; (174)], or following infection with the protozoan parasite *Trypanoplasma borreli* (175), illustrating the important role that TNFα plays in teleost host defense.

The proliferation and differentiation of immune cells is of great importance in host defense, since these processes replenish the immune cell pool that die throughout the course of an infection or during normal homeostatic processes. Haematopoesis and proliferation are influenced by the cytokines and growth factors to which cells are exposed. One of the most important growth factors of the mammalian immune system is macrophage colony stimulating factor-1, or M-CSF/CSF-1, a cytokine involved in the proliferation and survival of macrophages and monocytes (176). This growth factor is naturally produced under steady-state conditions, but may be quickly translated and secreted following stimulation with appropriate pathogens or mitogens. A receptor for this growth factor, CSF-1R, is expressed on the cells of the monocytic lineage and the expression level of this receptor increases as cells differentiate from monocytes to fully functional macrophages (177). In the goldfish, activity of CSF-1 is controlled through a number of mechanisms including gene expression, downstream intracellular signaling pathways, expression of CSF-1R, and the secretion of a soluble form of the CSF-1 receptor that competes with the membrane-bound form of the receptor for the cytokine CSF-1 (178).

Another protein involved in the regulation of macrophage haematopoesis in the goldfish is granulin. Granulin belongs to a group of highly conserved growth factors and, in goldfish, acts to induce the proliferation of early myeloid progenitors and monocytes (179). Not surprisingly, this growth factor is expressed at its highest levels in the head kidney, the primary haemotopoetic tissue of teleost fish, and is up regulated in proliferating macrophages (179).

Due to the important physiological role of the immune system, an animal's fitness is greatly impacted by its immunocompetence. Many studies have used various biomarkers of the immune system, or examined the overall immune response to a pathogen, to show deleterious effects of xenobiotic exposure (4; 6; 7). These xenobiotics may function to suppress the immune system through direct immunotoxic effects, through modification of the endocrine system, or by invoking a general stress response

that subsequently suppresses immune function. A large number of xenobiotics are present in municipal wastewater (10; 16; 65; 49) and reuse water produced through membrane UF (151). Despite this, the use of recycled water to replace the use of potable water sources for non-consumption use continues in many urban areas without a full impact assessment of the potential health concerns. If these xenobiotics are not successfully removed the potential exists for these chemicals to exert direct physiological effects in sensitive organisms, including humans.

In this chapter I report the results of experiments designed to examine the effect of exposing an aquatic vertebrate, goldfish (*Carassius auratus*), to either final treatment municipal wastewater (FE) or to reuse water produced through either membrane UF alone (MF wastewater), or MF wastewater that was subsequently passed through a granular activated carbon filtration unit (MCF wastewater). Peripheral blood leukocytes (PBLs) or kidney tissue was sampled from a number of fish prior to wastewater exposure and at 7, 21, 60 and 90 days following wastewater exposure during the Winter 2006 experiment. This was done in order to determine if municipal wastewater affected mitogen-stimulated PBL proliferation or the expression of a number of key immunological genes, as determined by semi-quantitative RT-PCR. Semi-quantitative RT-PCR was used rather than the ideal real-time PCR due to the large number of samples generated in the experiment (n=156 for each gene; 4 genes analysed) and the associated high financial costs.

6.2 *Results*

6.2.1 Peripheral blood leukocyte proliferation in goldfish exposed to different wastewaters (Winter 2006 experiment)

6.2.1.1 Non-stimulated Peripheral Blood Leukocyte Proliferation

Baseline, or non-stimulated proliferation of peripheral blood leukocytes (PBLs) was measured in goldfish exposed to municipal wastewater for various periods of time. In these fish, absorbance values (indicative of relative proliferation) were all very low (~0.1 OD units; Fig. 6.1) suggesting that exposure to different wastewater treatments did not induce a large, non-specific proliferative response in PBLs. Mean non-stimulated

absorbance values were analyzed using an analysis of variance test (3-way ANOVA using the sex of the fish, length of exposure, and wastewater treatment). Statistical analysis determined that neither the sex of the fish nor the wastewater treatment had an effect on the non-stimulated (baseline) PBL proliferation ($P>0.05$). However, the specific day the fish were sampled on as well as the interaction between wastewater treatment and the length of exposure was found to be statistically significant ($P<0.01$ and $P<0.001$, respectively).

Specifically, when analyzing the non-stimulated proliferation data for trends between treatment groups at each sampling time point (statistical significance indicated with differing lower case letters), the only statistically significant difference between wastewater treatment groups was on Day 90. On Day 90 post exposure, MCF wastewater exposed fish were found to have a significantly higher basal level of proliferation than Day 90 MF wastewater exposed fish ($P<0.01$, Fig. 6.1). The effect of the length of exposure within wastewater treatment groups on the baseline proliferative ability of PBLs was also analyzed (statistical significance indicated by differing upper case letters). No statistically significant changes in absorbance were observed in the majority of experiment groups (FE and MF, $P>0.05$). Some MCF wastewater exposed fish did have significantly higher non-stimulated PBL proliferative responses; Day 90 MCF wastewater exposed fish values were significantly greater than all earlier MCF wastewater time points ($P<0.01$ for all, Fig. 6.1). Day 7, 21, and 60 MCF wastewater exposed fish did not differ significantly from each other ($P>0.05$).

The mean absorbance value for Day 90 MCF wastewater exposed fish, the only non-stimulated experimental group to differ significantly from all other wastewater exposed groups, differed from other groups by only a relatively small amount (0.03 OD units from the mean of Day 90 MF fish). Since this statistically significant difference was only observed with fish exposed to MCF wastewater at Day 90, it is possible that this higher baseline absorbency was due to a higher proliferative response of one or two fish within the group because of heterogeneity within the fish population.

When each treatment group from each sampling period was compared against control baseline proliferation using a one-way ANOVA, all experimental groups had slightly higher mean absorbency values that nevertheless differed significantly from

control fish ($P<0.05$, statistical significance indicated with asterisks, Fig. 6.1), with the exception of Day 7 fish exposed to MCF wastewater ($P>0.05$). Since this slight upregulation was observed in nearly all exposure treatments and time points, it is unlikely that the small increase in non-stimulated PBL proliferation was due to xenobiotic exposure.

6.2.1.2 Mitogen Stimulated Peripheral Blood Leukocyte Proliferation

For each experimental group, the non-stimulated and mitogen-stimulated proliferation responses measured represent the same sample of fish for each treatment group and showed uniform consistency of non-stimulated PBL proliferation responses. Due to these reasons and the constraints of the statistical tests used, we chose to analyze trends in mitogen-stimulated PBL proliferation using a log transformed ratio between mitogen stimulated and non-stimulated groups. This method effectively eliminated any increase in absorbance value of an experimental group due to a higher initial number of seeded cells per well.

The proliferation of goldfish PBLs was examined as a fold-increase over the corresponding non-stimulated proliferation absorbance values (hereafter referred to as 'proliferation ratio'). Data were analyzed both between treatment groups for each experimental time point as well as within treatment groups over time. Similar to that of the non-stimulated proliferation, neither the gender of the fish nor the wastewater treatment had a significant effect on the proliferation ratio. The length of exposure ($P<0.0001$) as well as the interaction between the type of wastewater treatment and the length of exposure ($P<0.001$) was both highly significant in impacting the proliferation ratio. Overall, mitogen stimulation resulted in proliferation ratios ranging from 2.8 fold to 6.7 fold increases over non-stimulated leukocyte proliferation (Day 60 FE and Day 21 MF wastewater exposed fish, respectively, Fig. 6.2). Control fish had a mean 5.5 fold increase over non-stimulated proliferation absorbency values.

When examining for effects of wastewater within each sampling day, no statistically significant difference were observed between the PBL proliferation ratios of fish exposed to FE, MF or MCF wastewater on Day 7, 60 or 90 post-exposure ($P>0.05$;

Fig. 6.2; statistical significance indicated by differing lower case letters). On Day 21, fish exposed to MF wastewater had significantly higher proliferation ratios than fish exposed to MCF wastewater at the same sampling time point ($P<0.05$). The mean PBL proliferation ratio of fish exposed to FE wastewater at Day 21 did not differ from either that of those exposed to MF wastewater at Day 21 ($P>0.05$) or to MCF wastewater at the same time point ($P=0.08$; Fig. 6.2).

Each wastewater treatment group was also examined for changes within the group over the 90-day experimental period (statistical significance indicated with differing upper case letters). Fish exposed to FE wastewater seemed to initially have an increased proliferative ability on Day 21, which was reduced by Days 60 and 90 post-exposure (Fig. 6.2). Statistically, on Day 7 post exposure, fish exposed to FE wastewater were only significantly different from the greatly reduced Day 90 FE PBL proliferation ratio fish ($P<0.05$), and did not differ from the earlier Day 21 and Day 60 time points ($P>0.05$, Day 21; $P=0.08$, Day 60). Fish exposed to FE wastewater for 21 days had a significantly increased mitogen stimulated PBL proliferation than either fish exposed to FE wastewater for 60 or 90 days ($P<0.001$), but not compared to Day 7 FE fish ($P>0.05$). Day 60 and Day 90 FE fish had relatively low PBL proliferation and did not differ from one another statistically ($P>0.05$; Fig. 6.2).

Peripheral blood leukocytes from fish exposed to MF wastewater had a similar pattern of statistical significance compared to FE exposed fish (Fig. 6.2). Short-term exposure of fish to MF wastewater (Day 7) differed significantly from the later time point of Day 60 ($P<0.001$), but not from the Day 21 or Day 90 time points ($P>0.05$). Day 21 fish exposed to MF wastewater had a significantly higher proliferation ratio than the following Day 60 and Day 90 MF wastewater experimental groups ($P<0.001$ and $P<0.01$, respectively), but, surprisingly, not from Day 7 PBL proliferation ($P>0.05$; Fig. 6.2). The PBL proliferation of fish exposed to MF wastewater at Day 60 did not differ from those exposed to MF wastewater at Day 90 ($P>0.05$), but Day 60 MF wastewater exposed fish did have a proliferation ratio significantly lower than the first two time points ($P<0.01$ and $P<0.001$, respectively). By 90 days post exposure, the PBL proliferation ratio of fish exposed to MF wastewater was only significantly lower than that of fish exposed to MF wastewater sampled at Day 21 post-exposure ($P<0.01$). Proliferation of PBL from fish

exposed to MCF wastewater did not differ across the ninety day experimental period ($P>0.05$).

The average proliferation ratio for each wastewater treatment group on each sampling day was compared to that of the control fish using a one-way ANOVA (statistical significance indicated with an asterisk). The proliferation ratio of all Day 7 and Day 21 treatment groups did not differ from the controls ($P>0.05$; Fig. 6.2). Interestingly, the proliferation of PBLs from fish exposed to FE, MF and MCF wastewater treatments at Day 60 and 90 all were statistically lower than control fish ($P<0.01$ for all except Day 60 MCF wastewater, where $P<0.05$), indicating a possible slight suppressive effect of long-term wastewater exposure.

6.2.2 Expression of immunological genes in the head kidney of goldfish exposed to different wastewaters

Goldfish mRNA was isolated from the head kidney of fish exposed to municipal sewage effluent (FE wastewater) or reuse water (MF or MCF wastewater) and converted into cDNA using reverse transcriptase. The level of gene expression of select immunological genes in each group of fish were compared to the expression level of a control gene, β-actin, both between treatment groups at each sampling time point and within a treatment group over the ninety day exposure period, as described for the PBL proliferation assay.

6.2.2.1 Expression of toll-like receptor-22

When the relative TLR-22 gene expression was compared between male and female fish for each treatment group and sampling times, no discernable differences were observed between the sexes ($P>0.05$). When male and female fish from each sampling point and wastewater treatment were grouped together, it was found that TLR-22 gene expression did not differ between any treatment groups on Day 7, 60 or 90, nor over the ninety day experimental period for fish exposed to FE or MCF wastewater treatment ($P>0.05$; Fig. 6.3). Fish exposed to MF wastewater for 21 days experienced a significantly higher level of TLR-22 gene expression than Day 21 fish exposed to MCF wastewater (statistical significance indicated by differing lower case letters; $P<0.05$), fish exposed to MF wastewater at Day 7 and 60 exposure time points (statistical significance

indicated with differing upper case letters; $P<0.05$), and control fish (statistical significance indicated with an asterisk; $P<0.05$; Fig. 6.3). Upon closer examination of the data, the TLR-22 gene expression of two female fish exposed to MF wastewater at Day 21 was extremely high compared to the remaining 10 fish (greater than three fold higher expression, as indicated by densitometry analysis). Furthermore, one fish from the FE treatment group exhibited greater than four-fold TLR-22 gene expression compared to the remaining eleven FE fish. However, when these fish were removed from the analysis, Day 21 fish exposed to MF wastewater still exhibited significantly higher TLR-22 gene expression than those fish exposed to MCF wastewater and sampled at Day 21 (statistical significance shown by differing lower case letters; $P<0.05$), Day 7 fish exposed to MF wastewater (statistical significance shown by differing upper case letters; $P<0.01$) and control fish (statistical significance indicated by asterisks; $P<0.001$; Fig. 6.4). No other wastewater treatment groups or exposure time points average TLR-22 gene expression values differed from one another or that the control fish values (Fig. 6.4).

6.2.2.2 Expression of tumor necrosis factor-α

The pro-inflammatory cytokine tumor-necrosis factor alpha (TNFα) is involved in a number of innate and acquired immune responses. As such, TNFα was examined using RT-PCR for changes in the mRNA expression levels following exposure to wastewater for various periods of time. As observed for TLR-22, gender did not impact the gene expression in goldfish exposed to municipal wastewater or recycled water ($P>0.05$). Interestingly, a significant increase in gene expression was found in the kidney from fish exposed to wastewater that contained the greatest concentrations of xenobiotics at Day 7 post-exposure (FE and MF wastewater) compared to wastewater that did not contain these relatively high concentrations of xenobiotics (MCF wastewater or control fish; Fig. 6.5). When examining the data for differences between wastewater treatment groups at each time point, fish exposed to both FE and MF wastewater for only seven days were found to have significantly higher TNFα gene expression than that of their MCF wastewater exposed cohorts ($P<0.01$; statistical significance indicated by differing lower case letters) and that of control fish ($P<0.001$; statistical significance indicated by asterisks; Fig. 6.5). This increase in TNFα mRNA expression did not persist at any of

the following time points (statistical significance within wastewater treatment groups over time indicated by differing upper case letters; Fig. 6.5).

6.2.2.3 Expression of colony stimulating factor-1 receptor

Expression of the receptor for macrophage colony stimulating factor-1 (CSF-1R) was also analyzed in goldfish kidney tissue. Wastewater exposure influenced the expression of CSF-1R mRNA in a similar manner between both male and female fish ($P>0.05$). Xenobiotic exposure, either through FE or MF wastewater water, resulted in an increased level of CSF-1R gene expression compared fish exposed to MCF wastewater ($P<0.001$; statistical significance indicated by differing lower case letters) or control fish ($P<0.001$; statistical significance indicated by asterisks) at both Day 7 and Day 21 post-exposure sampling time points (Fig. 6.6). This induction effect was not observed at later time points; fish exposed to FE and MF wastewater for sixty and ninety days did not differ from either fish exposed to MCF wastewater or control conditions ($P>0.05$).

6.2.2.4 Expression of granulin

The gene expression of the haematopoetic myeloid progenitor growth factor, granulin, was determined in fish exposed to FE, MF or MCF wastewater treatments for up to ninety days. Even though both granulin and CSF-1R have been shown to be involved in the proliferation and development of monocytes (176; 179), a suppression of granulin mRNA was detected in goldfish tissue at Day 7 post-exposure; this reduction in gene expression occurred to an equal degree in male and female fish ($P>0.05$) of all wastewater treatment groups (Fig. 6.7). This is in contrast to the observed elevation in CSF-1R gene expression in the same tissue isolated from the same individual fish (Fig. 6.6). At no individual time point tested did the level of gene expression of granulin differ between wastewater treatment groups ($P>0.05$; statistical significance indicated by differing lower case letters; Fig. 6.7). When analyzing the relative granulin mRNA expression over time, fish exposed to FE wastewater experienced a significant suppression of granulin mRNA expression at Day 7 post-expression, but by Day 21 and

all time points following this gene expression had returned to constitutive expression levels ($P<0.001$; statistical significance indicated by differing upper case letters; Fig. 6.7). A similar pattern of gene expression was observed in fish from the MF wastewater treatment. Following the observed suppression of granulin mRNA at Day 7, the expression of this gene increased in a consistent manner until Day 60 post-exposure, and this constitutive expression was evident until Day 90 (Fig. 6.7). Fish exposed to MCF wastewater also experienced a consistent increase in granulin expression, reaching the highest relative gene expression by Day 90 post-exposure (Fig. 6.7).

6.3 Discussion

This chapter contains results of experiments whose aims were to investigate the possible effect of different wastewater treatments on various immune parameters of the goldfish, namely mitogen-stimulated peripheral blood leukocyte proliferation and the relative expression level of key immunological genes in the fish head kidney.

Many studies have focused on the effect of municipal sewage treatment plant effluent on the immune system of teleost fish (134; 133; 135; 128; 180). Rainbow trout (*Oncorhynchus mykiss*) exposed to diluted municipal sewage effluent for twenty-seven days had enhanced mitogen-stimulated peripheral blood lymphocyte proliferation compared to control fish, even though effluent exposure decreased the number of lymphocytes in the blood (128). Alternatively, Katuka (133) found that the numbers of granulocytes and lymphocytes in the blood from goldfish exposed to 20% treated sewage effluent were increased seven days post-exposure compared to control fish. By thirty days post-exposure however, the levels of blood granulocytes and lymphocytes, as well as the phagocytic cell activity of effluent exposed fish were suppressed compared to controls. While we observed changes in the mitogen-stimulated proliferative ability of PBL, it is unknown whether the numbers of leukocytes varied over time.

Though the effect of municipal wastewater exposure on leukocyte proliferation on fish immune response has been examined (as described above), the majority of immunotoxicological studies have involved the *in vivo* or *in vitro* exposure of fish to specific xenobiotics (4; 6-8). For example, Carlson *et al.* (30) injected Japanese medaka (*Oryzias latipes*) with 2 μg/g body weight benzo[a]pyrene and observed a significant

reduction in *in vitro* mitogen-stimulated proliferation of B and T lymphocytes one week later.

Although we did not observe substantial wastewater treatment-specific effects on the proliferation ratios of goldfish PBLs in this study, other aspects of the immune response may be impacted by xenobiotic exposure. In order to examine this we used semi-quantitative RT-PCR to analyze the expression of known immunological or haematopoetic genes in the kidney of goldfish exposed to different wastewater treatments for up to ninety days. The genes selected have been shown to be important in the pro-inflammatory immune response (170; 172; 173), as well as haemaotopoesis and development of immune cells (176; 179), both crucial processes if an appropriate immune response upon pathogen challenge is to occur.

Several studies have demonstrated changes in the expression level of hundreds of genes associated with the immune response and physiological function in fish following pollutant exposure (164; 31; 167; 168; 165; 169; 166). While the majority of the studies conducted have primarily focused on genes involved in metabolism, xenobiotic exposure (for example, CYP1A), or biomarkers of EDCs such as Vtg, others have also examined genes with direct immunological relevance (31; 167; 166). We also examined the expression levels of immunologically relevant genes, namely toll-like receptor-22, tumor-necrosis factor alpha, macrophage colony stimulating receptor-1 and granulin.

Toll-like receptor-22 (TLR-22) gene expression did not differ greatly between the wastewater treatment groups, nor did it change with the length of exposure. A possible exception to this pattern occurred on Day 21 post-exposure; xenobiotic exposure in the MF wastewater treatment may have resulted in an increased TLR-22 gene expression compared to other wastewater exposures or time points. Since TLRs recognize specific pathogen surface patterns and are important receptors in the recognition of self from non-self, one would expect to see a constitutive level of expression of this gene. On Day 21, a small proportion (~12%) of FE and MF exposure fish had greatly enhanced TLR-22 mRNA expression compared to the majority of cohort fish. Due to the fact that this large variability was not observed in wastewater treatment groups at any other sampling points, it is likely that the increase in average TLR-22 gene expression was due to heterogeneity between fish in endogenous TLR-22 expression levels, or possibly due to prior bacterial

infections in these specific fish. Previous work in our lab (171) found that *in vitro* exposure of goldfish macrophages to pathogens or to bacterial products such as LPS resulted in an induction of the TLR-22 gene compared to untreated macrophages, indicating a potential for this gene's up-regulation to be in response to bacterial stimuli. The exact ligand and function of TLR-22 is thus far unknown.

Interestingly, an increase in the transcription of the pro-inflammatory cytokine tumor-necrosis factor alpha (TNFα) gene was observed following short-term exposure to xenobiotics present in either the FE wastewater or the MF wastewater. TNFα mRNA was up-regulated approximately five-fold compared to fish that were not exposed to xenobiotics, control fish, and fish exposed to MCF wastewater. This up-regulation was not due to a handling or transport related stress response, as fish exposed to MCF wastewater did not show this induction. If this increase in TNFα mRNA was followed by a corresponding increase in TNFα secretion from leukocytes with no reduction in TNFα receptor prevalence, fish exposed to FE and MF wastewater may have experienced a transient period of immunostimulation and an increased pro-inflammatory response at seven days post-exposure. However, whether the increase in TNFα mRNA expression did indeed correlate with an increase in the actual cytokine secretion remains to be determined. At twenty-one days post-exposure and all time points thereafter, TNFα gene expression in all wastewater treatment groups was similar to that of control fish. The return to constitutive levels of TNFα gene expression could be due to homeostatic mechanisms that stabilized any immunostimulatory effects caused by exposure to xenobiotics in the effluent. Alternatively, due to the labile nature of the chemical compounds and concentrations of xenobiotics found in the wastewater, it is possible that the chemical factors which induced the increase in TNFα gene expression were no longer present for the remaining sampling time points.

Quabius *et al.* (167) exposed immature female rainbow trout (*O. mykiss*) head kidney leukocytes to PCB 126 and also found a transient increase in the expression of a pro-inflammatory cytokine, IL-1β. The expression of IL-1β was found to be significantly up-regulated between 2-4 hours following exposure to this xenobiotic; however, PCB exposure was not found to cause any permanent damage to these immune cells (167). In the same study, chronic stress mimicked by prolonged exposure to the hormone cortisol,

inhibited IL-1β gene expression in addition to reducing the level of CYP1A gene expression, indicating that extended periods of stress in these animals may not only result in immunosuppression, but also an impaired ability to metabolize toxicants, further reducing the animal's overall fitness.

While an increased TNFα mRNA expression has been shown in fish monocytes following the addition of a mitogen or parasite (174; 175) and following exposure to FE or MF reuse water in this study, exposure of fish or fish tissues to xenobiotics usually result in immunosuppressive effects (27; 29; 30; 44; 181; 123; 31). For example, when carp macrophages were isolated and exposed *in vitro* to the pesticide and wood preservative pentachlorophenol (PCP), a suppression of TNFα and IL-1β mRNA was observed in exposed macrophages compared to unexposed controls (31). Furthermore, supernatants from these PCP treated macrophages had a reduced stimulatory effect on B cells, as shown by a significantly suppression of IgM secretion (31).

Similar to TNFα gene expression, exposure of goldfish to wastewater contaminated with the highest concentration of xenobiotics resulted in an induction of colony-stimulating factor-1 receptor (CSF-1R) kidney mRNA over that of control fish or those exposed to MCF wastewater. CSF-1R gene expression in fish exposed to MCF wastewater did not differ from that of control fish at any time point. Unlike TNFα however, CSF-1R expression was elevated until Day 21 post-exposure in fish exposed to FE and MF wastewater. The increase in CSF-1R mRNA may be correlated to an increased prevalence of CSF-1R protein on the surface of kidney macrophages, indicating that wastewater exposure may have caused an increase in the secretion of the receptor's native ligand, CSF-1. A higher level of ligand-CSF-1R binding could have resulted in an induction of receptor-mediated downstream events in these cells, such as the proliferation, differentiation and survival of macrophages and cells of the monocytic lineage (177). Consequently, the increase in the transcription of the CSF-1R gene may correspond to a concomitant increase in the number of proliferating and developing monocytes or macrophages within these fish exposed in fish exposed to FE and MF wastewater. The observed increase in the proliferation ratio of PBLs from fish exposed to Day 21 MF wastewater compared to that of Day 21 MCF wastewater exposed fish may have been partially due to the induction of the CSF-1R gene. Alternatively, it is possible

that xenobiotic exposure resulted in a low level of CSF-1 in the tissues of the fish and the CSF-1R gene was upregulated because of homeostatic mechanisms rather than as a direct result of xenobiotics acting upon the CSF-1R gene itself. However, since neither CSF-1R protein expression nor CSF-1 mRNA were measured, it is impossible to know definitively what specific effects the increased CSF-1R gene expression may have had on goldfish immune response.

The gene expression of granulin, another growth factor involved in the proliferation of myeloid progenitor cells, was very different compared to the other immunological genes examined. While wastewater treatment did not seem to affect the mRNA expression of this growth factor, the length of exposure did result in substantial variation between sampling dates. All wastewater exposure treatments (FE, MF and MCF) exhibited a marked decrease in kidney granulin gene expression at Day 7 after wastewater exposure. However, gene expression was similar to that of control fish for all other time points sampled. If the observed reduction in granulin gene expression was mirrored by a corresponding reduction in the level of granulin secretion and its effecter function, it is possible that the proliferation of early monocytic progenitor cells or monocyte subpopulations could have been suppressed in the kidney of wastewater exposed fish, as studies have shown that exposure of goldfish primary head kidney myeloid progenitor cells to recombinant goldfish granulin induced a dose dependant proliferation of these cells (179).

The reduction in granulin mRNA expression observed on Day 7 post-exposure was unlikely to be due to xenobiotics present in the wastewater, since this suppression was detected in all treatment groups, including fish exposed to MCF wastewater. Of the chemicals we detected, passage through a GAC filter unit substantially reduced xenobiotic contamination of the wastewater. However, wastewater effluent potentially contains thousands of different xenobiotic chemical compounds (1) and chemical analysis of wastewater through gas chromatography/mass spectrometry is limited in the chemicals that may be accurately detected. It is possible that the suppression of granulin mRNA expression in exposed goldfish may be due to a xenobiotic(s) that was not sufficiently removed by the activated carbon filter unit, and remained undetected due to our chemical analysis protocols.

Granulin gene expression may have also been reduced due to persistent stress response caused by transport from the University of Alberta aquatics facility to Gold Bar seven days beforehand that only affected granulin gene expression, or due to exposure to higher concentrations of organic compounds such as ammonia or nitrate/nitrite. During the Winter 2006 study, ammonia levels in FE tanks ranged up to 14.15 mg/L nitrogen, whereas MF and MCF wastewater ammonia levels were less variable (0.16 - 3.79 mg/L nitrogen; see Chapter Four). Exposure of aquatic organisms such as fish to ammonia can affect their physiology and immune response. For example, smolting Chinook salmon (*O. tshawytscha)* exposed to ammonia (up to 10 mg/L nitrogen for 10 days) had significantly fewer circulating immune cells such as lymphocytes and granulocytes compared to control fish, as well as a higher mortality upon subsequent infection with the bacteria *Vibrio anguillarum* (182). In my study, goldfish exposed to FE wastewater experienced periods of up to eight days in which measured ammonia levels exceeded 9 mg/L nitrogen (January 29 to January 26, 2006; data not shown). The potential impacts of ammonia in the wastewater on other aspects of the study are not known.

In toxicological studies, it is important to use a number of immunological assays to best determine the effects of xenobiotic exposure on aquatic organisms' immune responses. *In vitro* immune function assays where leukocytes or other factors are directly isolated from exposed fish are advantageous in that they measure the cumulative effect of multiple-xenobiotic exposure on fish immune response. In this study, the peripheral blood leukocyte proliferation assay examined the ability of these cells to proliferate in response to mitogens, but this facet of the goldfish immune system was largely unaffected by wastewater treatment. In previous experiments done during the Spring/Summer 2005, I used other immunocompetence assays such as the respiratory burst and nitric oxide assay to assess the impacts of wastewater treatment on fish immune responses. These studies used *in vitro* cultured primary head kidney macrophages that were isolated from fish exposed to FE, MF or MCF wastewater for various time periods. However, no differences were observed between treatment groups or over time (data not shown); it is hypothesized that this was due to the six to eight day culturing period between the isolation of the cells and the application of the assay. The respiratory burst

and nitric oxide assays were not used on macrophages or PBLs directly isolated from exposed fish in the Winter 2006 experiment due to time constraints.

I believe that the expression of key immunological genes by semi-quantitative RT-PCR may be used to help determine the mechanisms that mediate xenobiotic-induced changes in the immune system. However, it may be difficult to predict the physiological effect on the whole organism by looking at mRNA expression alone, as protein expression and ligand/receptor production and display may also greatly influence downstream effects. Furthermore, the initiation, maintenance and suppression of an appropriate immune response is a complex process that involves numerous cytokines and growth factors, many interactions of which have yet to be fully elucidated.

Immunosuppression of fish exposed to xenobiotics, either as individual chemicals or as complex mixtures found in effluent, have been reported by numerous researchers (4; 7). These effects range from the suppression of antibody production or B cell numbers (27; 29; 30; 181; 123; 31), changes in the production of reactive oxygen intermediates (181; 31; 183), cell proliferation (133; 30; 44; 128), phagocytic ability (181), and the expression of genes involved in an immune response and inflammation (31; 184). In the present study, we observed indications of a possible acute stimulatory effect in the immune system of goldfish exposed to xenobiotics, either in FE or MF wastewater. These responses were generally eliminated if wastewater was filtered using activated carbon. Many toxicological experiments involve the exposure of organisms to pollutant or EDCs in the range of days to weeks. Consequently, transient increases in gene expression, as observed in Quabius *et al.* (167), or even the TNFα induction seen in my study following seven days of FE and MF wastewater exposure, may be undetected due to the sampling time points chosen. The temporal differences observed within wastewater treatment groups over the ninety day exposure period further highlight the importance of using both short-term as well as long-term exposure periods to best determine what, if any, physiological effects wastewater exposure has on the immune responses of aquatic organisms.

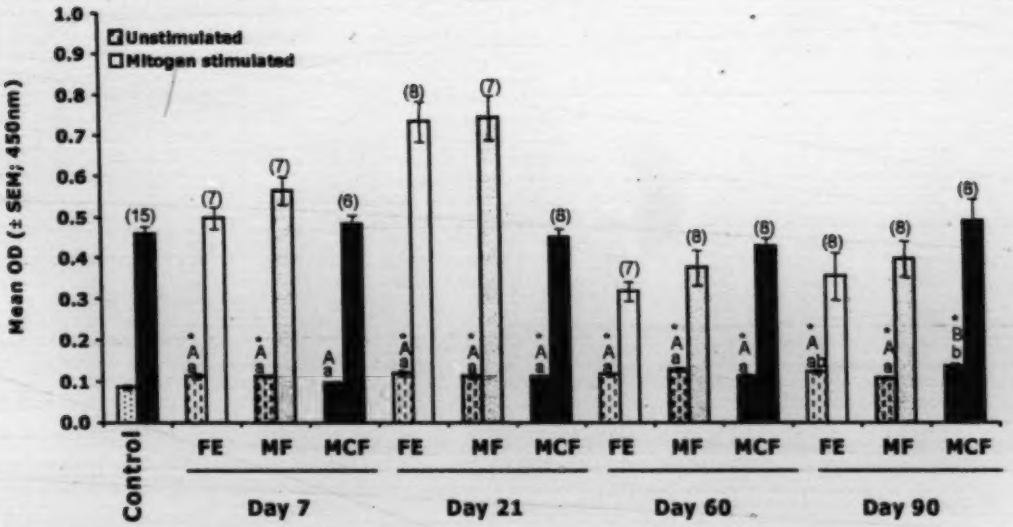

Figure 6.1 Non-stimulated and stimulated peripheral blood leukocyte (PBL) proliferation in goldfish exposed to different wastewater treatments during the Winter 2006 experiment. PBLs, isolated from the blood of the fish, were seeded in sterile 96 well plates (50,000 cells per well), in addition to either complete medium alone (non-stimulated) or an equal volume of mitogens (10 µg/mL ConA; 10 ng/mL PMA; 100 ng/mL calcium ionophore A23187) and cultured for 48 hours at 20°C. BrdU labelling reagent was then added and cells were allowed to proliferate for a further 24hrs prior to colourimetric analysis, as described in Chapter 3. Sample size, as indicated on the graph, refers to both non-stimulated and stimulated bars (samples were paired). Differing lower case letters indicate statistical significance between treatment groups within the same sampling day, differing upper case letters refer to significant differences within a wastewater treatment group over time, and asterisks refer to significant differences between experimental groups and control fish.

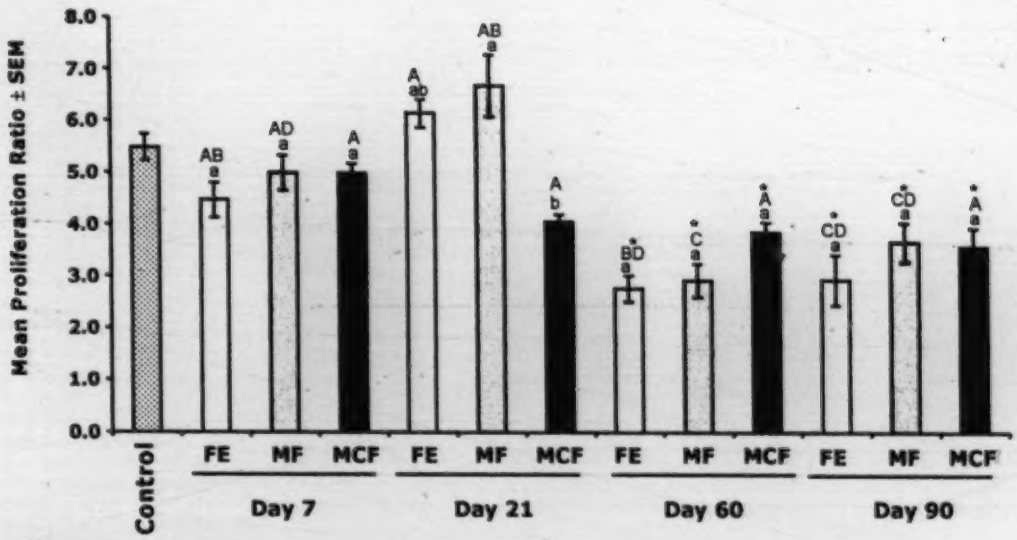

Figure 6.2 Proliferation Ratio of peripheral blood leukocytes (mean mitogen stimulated absorbance/mean non-stimulated absorbance at 450nm) isolated from fish exposed to different wastewater treatments during the Winter 2006 experiment. Sample size, data and methodologies are as described in Figure 6.1. Differing lower case letters indicate statistical significance between treatment groups within the same sampling day, differing upper case letters refer to significant differences within a wastewater treatment group over time, and asterisks refer to significant differences between experimental groups and control fish.

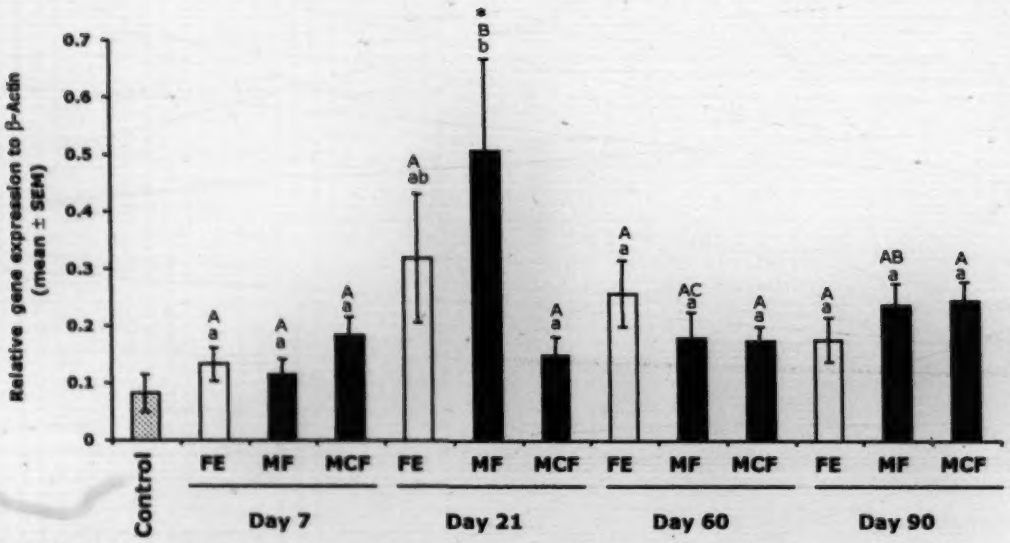

Figure 6.3 Semi-quantitative RT-PCR gene expression of toll-like receptor 22 in the head kidney of fish exposed to different wastewater treatments during the Winter 2006 experiment. Relative expression levels of the specific gene bands were determined by densitometry using the Kodak ID 3.0 software (Eastman Kodak). Letters/symbols indicating statistical significance are as described in Figure 6.1 Sample size is 12 fish for all except: Day 7 MCF (n = 10) and Control, Day 60 FE, Day 60 MF, Day 90 FE, Day 90 MF (n = 11).

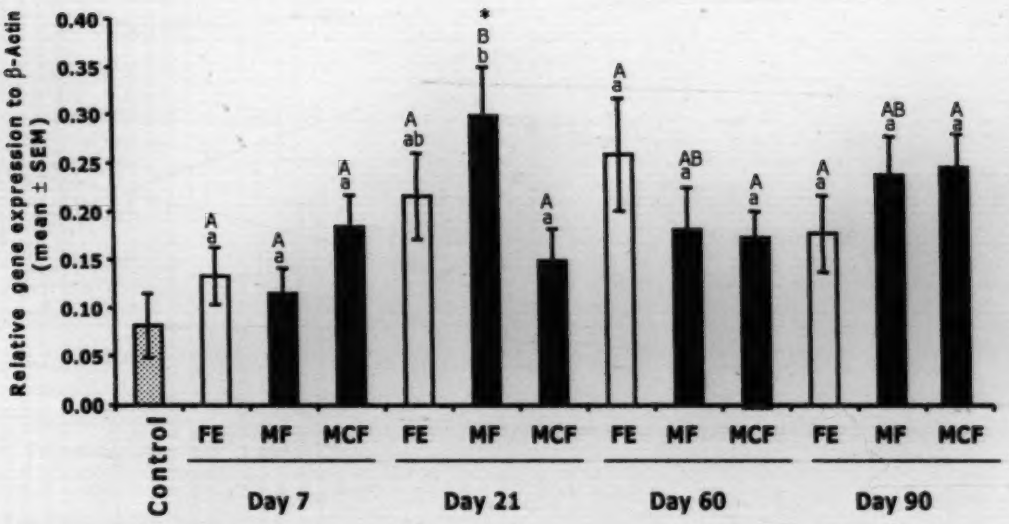

Figure 6.4 Semi-quantitative RT-PCR gene expression of toll-like receptor 22 in the head kidney of fish exposed to different wastewater treatments during the Winter 2006 experiment upon removal of fish densitometry data that were greater than 3 fold that of their remaining cohorts. Relative expression levels of the specific gene bands were determined by densitometry using the Kodak ID 3.0 software (Eastman Kodak). Letters/symbols indicating statistical significance are as described in Figure 6.1. Sample size is 12 fish for all except: Day 7 MCF and Day 21 MF (n = 10 for each) and Control, Day 21 FE, Day 60 FE, Day 60 MF, Day 90 FE, Day 90 MF (n = 11).

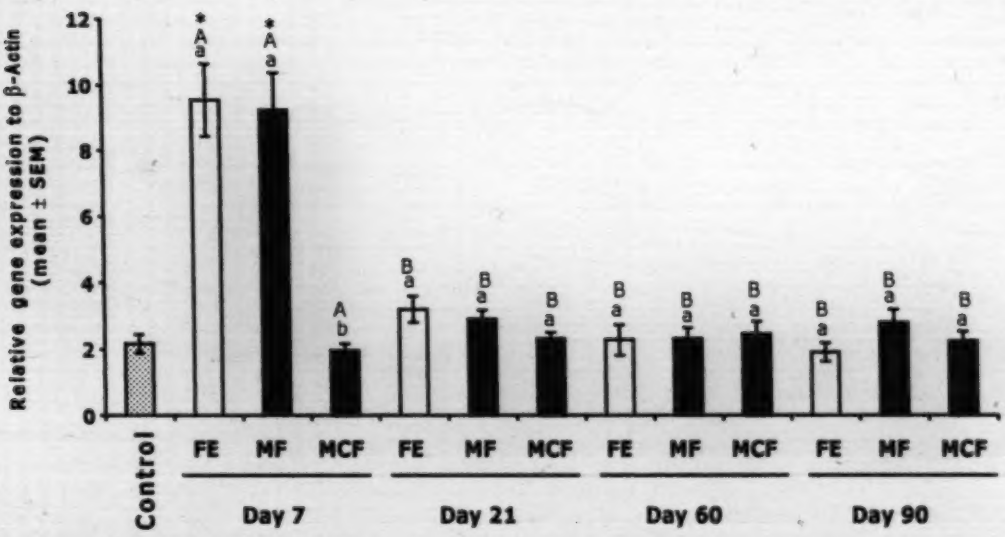

Figure 6.5 Semi-quantitative RT-PCR gene expression of tumor necrosis factor-alpha in the head kidney of fish exposed to different wastewater treatments during the Winter 2006 experiment. Relative expression levels of the specific gene bands were determined by densitometry using the Kodak ID 3.0 software (Eastman Kodak). Letters/symbols indicating statistical significance are as described in Figure 6.1 Sample size is 12 fish for all except: Day 7 MCF (n = 9) and Control, Day 7 MF, Day 21 FE, Day 60 FE, Day 90 FE and Day 90 MF (n = 11).

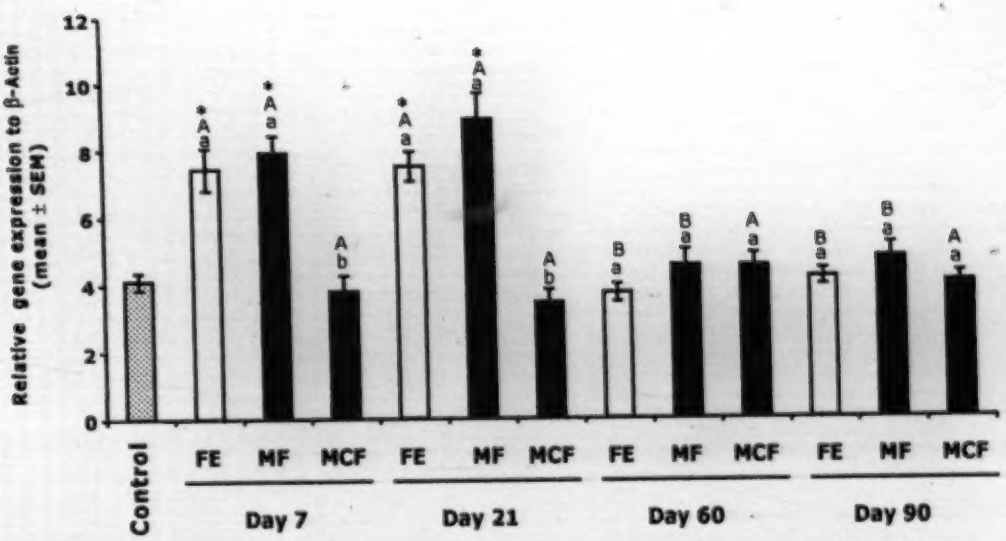

Figure 6.6 Semi-quantitative RT-PCR gene expression of colony stimulating factor receptor-1 in the head kidney of fish exposed to different wastewater treatments during the Winter 2006 experiment. Relative expression levels of the specific gene bands were determined by densitometry using the Kodak ID 3.0 software (Eastman Kodak). Letters/symbols indicating statistical significance are as described in Figure 6.1 (n=12).

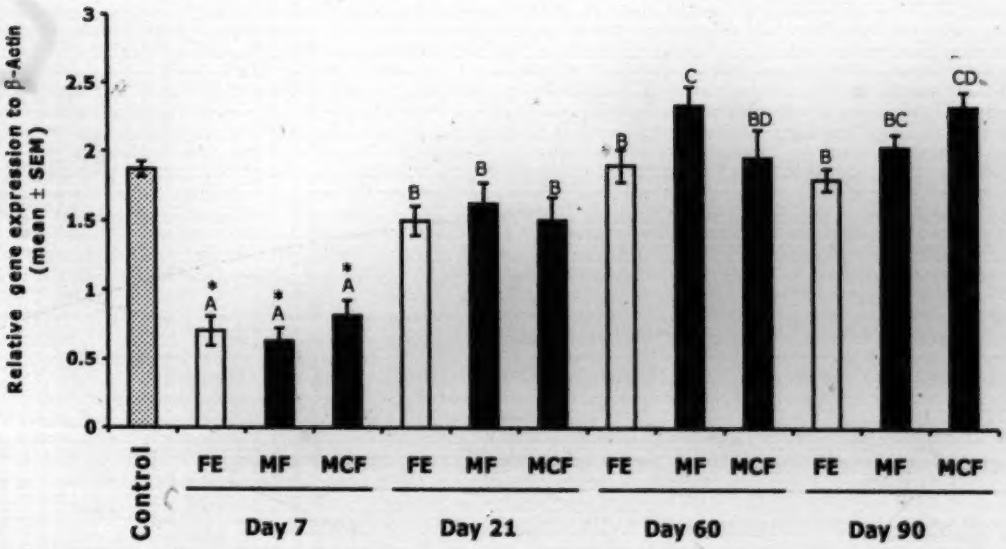

Figure 6.7 Semi-quantitative RT-PCR gene expression of granulin in the head kidney of fish exposed to different wastewater treatments during the Winter 2006 experiment. Relative expression levels of the specific gene bands were determined by densitometry using the Kodak ID 3.0 software (Eastman Kodak). Letters/symbols indicating statistical significance are as described in Figure 6.1 (n = 12).

CHAPTER SEVEN

TRYPANOSOMA DANILEWSKYI INFECTION OF FISH EXPOSED TO WASTEWATER

7.1 Introduction

Pathogen infection studies, in which organisms are exposed to live parasites, bacteria or other microorganisms *in vivo*, directly measure the ability of the host immune system to control or resolve an infection. This method allows for a much more comprehensive assessment of an animal's immunocompetence than assays that measure single, cellular-based functions alone, such as reactive oxygen intermediate production, cytokine secretion or the expression of immune genes. This is because cellular-based assays may not necessarily predict the net effects of an exposure treatment on the animal's physiology due to redundancy within the organisms' immune system and/or physiological homeostatic mechanisms.

Trypanosoma danilewskyi, the parasite used for the goldfish infection studies discussed in this chapter, is a haemoflagellate protozoan that can be naturally found in the blood of freshwater cyprids such as the common carp (185). In the wild, *T. danilewskyi* is naturally transmitted between fish by blood-feeding leeches of the genera *Hemiclepsis* and *Pisciola* (186; 187). The parasites first develop within the stomach of their intermediate host and then migrate to the crop where they infect the fish during the leeches' next feeding. Parasites replicate within the blood of the fish, with parasitemia generally peaking in experimentally infected goldfish approximately fourteen to twenty-one days post-infection (138; 139). By fifty-six to eighty-four days post-infection, parasitemia levels are greatly reduced. Previously infected fish are resistant to re-infection and it is believed that this immunity is due to anti-parasite antibodies, as inoculation of naïve fish with plasma from fish that have recovered from previous *T. danilewskyi* infection confers protection upon the naïve fish (185; 138; 188).

Goldfish are closely related to carp and are also susceptible to *T. danilewskyi* infection (138). Goldfish can be infected through either intra-peritoneal infection of parasites isolated from previously infected fish, or by injection with parasites grown using *in vitro* cultivation (138; 139). Cultivation and maintenance of parasite populations

using *in vitro* methodologies is advantageous as large numbers of these parasites (primarily trypomastigotes) can be grown quickly and inexpensively with no associated fish mortalities.

Many aquaculture operations are located on river systems that are also the recipient of municipal and industrial effluents or agricultural runoff. Since many farmed fish populations commonly experience bacterial or parasitic infections (189; 190) and exposure of aquatic vertebrates to xenobiotics or endocrine disrupting compounds may result in immunosuppression (4; 6; 7), it is possible that exposure of farmed or native fish populations to polluted environments may exacerbate pathogenic infections.

Studies have demonstrated the interaction between pollutant exposure and parasite or bacterial infection in host resistance and physiology. For example, cadmium exposure has been shown to reduce survival of three-spined sticklebacks (*Gastersteus aculeatus)* infected with the tapeworm *Schistocephalus solidus* compared to the survival of cadmium-exposed non-infected fish (191). Enhanced pathogenicity was thought to be due to immunosuppressive effects induced by xenobiotic exposure. Exposure of Japanese medaka to benzo[*a*]pyrene reduces host resistance against the bacterial pathogen *Yersinia ruckeri*, and is associated with reduction of both mitogen-stimulated lymphocyte proliferation and phagocyte mediated oxygen radical production (30). Pollutants may not simply immunosuppress hosts and render them more susceptible to infection. Indeed, the parasitism itself may exacerbate the toxic effects of environmental pollutants such that the health of the parasitized organism is reduced beyond that of unparasitized contaminant-exposed hosts. Marcogliese *et al.* (192) demonstrated that the combination of both pollutant exposure and nematode or digenean worm infection in yellow perch (*Perca flavencens*) resulted in greater oxidative stress than that observed in unparasitized fish from the same polluted location.

In this chapter I examined the effect of exposing goldfish to treated final sewage effluent (FE wastewater) on the course of infection of the protozoan parasite *Trypanosoma danilewskyi.* Also of interest was whether further treatment of this wastewater through membrane ultrafiltration (UF; MF wastewater) or subsequent granular activated carbon (GAC) unit filtration (MCF wastewater) reduced any potential effects of FE wastewater exposure on parasitemia of the exposed fish. Goldfish were

exposed to wastewater effluents on two separate occasions: The first parasite infection experiment (termed Winter 2006) occurred between February 5, 2006 and April 30, 2006 and involved the infection of control goldfish or goldfish exposed to municipal wastewater for twenty days with 6.25×10^6 parasites per fish. The second parasite experiment (termed Summer 2006) involved the infection of control goldfish or fish that had been exposed to wastewater for approximately seven days with either 6.25×10^6 parasites per fish or a higher dose of 1.0×10^7 parasites per fish and occurred between June 1, 2006 and August 24, 2006. For each parasite infection study, fish were monitored for mean parasitemia and survivorship three days post-infection and on a weekly basis until approximately eighty-four days later.

7.2 Results

7.2.1 *T. danilewskyi* infection of fish exposed to different wastewaters (Winter 2006 experiment)

In the Winter 2006 experiment all fish exposed to FE wastewater died accidentally on Day 24 post-infection due to a valve malfunction that resulted in the tank draining of water. Consequently, for all following time points only fish exposed to MF wastewater were exposed to xenobiotics in the wastewater. This is due to the incomplete removal of various pharmaceutical residues and pesticides from municipal sewage effluent by the membrane UF (see Chapter 4). Statistical analysis on the effect of wastewater treatment (MF, MCF and BS water), fish gender, and the number of days post infection on mean parasitemia of *T. danilewskyi* infected goldfish resulted in no significant effects of wastewater treatment alone or the sex of the fish on parasitemia ($P>0.05$). However, the number of days post-infection was found to be highly significant ($P<0.01$) as would be expected in an infection study in which some fish are able to clear an infection over time. Furthermore, the interaction between the number of days post-infection and the wastewater treatment was also found to be highly significant ($P<0.01$), indicating that parasitemia levels in the blood of fish exposed to certain wastewater treatments differed significantly from that of fish exposed to other wastewater treatments, but only at certain time points of the infection.

When examining parasitemia trends in fish during the early stages of infection (3-28 days post-infection) no substantial differences were observed in fish exposed to wastewater containing xenobiotics (FE, MF wastewater) and those exposed to environments with low to zero levels of xenobiotics (MCF wastewater; Fig. 7.1). During the mid-stages of infection (Days 35-49 post-infection), fish exposed to MF wastewater maintained a mean parasitemia of ten fold higher than that of fish not exposed to xenobiotics (MCF wastewater, BS water; Fig.7.1). However, due to the variability between fish within each treatment group during Days 35-49 post-infection, it is difficult to determine if xenobiotic exposure resulted in true differences between infected exposure groups. As the experiment progressed, both fish exposed to MCF wastewater and BS water successfully reduced their mean parasitemia to low levels, whereas fish exposed to the MF wastewater were unable to clear the infection and parasite concentrations within the blood of these fish remained high (Fig. 7.1). Furthermore, fish exposed to MF wastewater experienced a substantial increase in mortality compared to fish exposed to other wastewater treatments: by Day 84 post-infection only 30% of fish exposed to MF wastewater remained alive, compared to greater than 80% survivorship of fish exposed to MCF wastewater and BS water (Fig. 7.2).

7.2.2 *T. danilewskyi* infection of fish exposed to different wastewaters (Summer 2006 experiment)

The second parasite infection experiment was done during the summer of 2006. The experimental design was similar to the previous experiment, except that in addition to the normal dose parasite exposure (6.25×10^6 parasites/fish) fish were also infected with a high dose (1.0×10^7 parasites/fish). In this study the gender of the fish, the number of parasites in the initial infection, and the wastewater treatment did not affect parasitemia ($P>0.05$). Consequently, the data for male and female fish from both normal and high infection parasite dose groups were combined for analysis. A significant effect of the number of days post-infection on parasite concentration was observed ($P<0.05$). This is expected, as the majority of infected fish initially had a high parasitemia in early stages of the infection but subsequently cleared the parasites. The interaction between the number of days post-infection and the initial parasite dose was also found to be significant ($P<0.05$), indicating that fish infected with different doses of parasites had

variable parasitemia levels depending upon the number of days following infection, but these trends were not consistently observed during the course of infection. The interaction between the number of days post-infection and the wastewater treatment was marginally significant ($P = 0.07$). This implies that on certain time points following parasite challenge, fish from different wastewater treatment groups may have had a lower concentration of parasites in their blood than other groups, and though this difference was not statistically significant, the reduction in parasitemia may be of biological relevance for the well being of the infected fish.

In the early stages of the infection (Days 3-21), the mean parasitemias in fish exposed to FE or MCF wastewater and BS water were similar (Fig. 7.3). Fish exposed to MF wastewater, however, had 10-50 fold lower parasitemia compared to other experimental groups. During the mid-stages of the infection (Days 21-42), while the parasitemia in fish exposed to MCF wastewater and BS water were similar, both goldfish exposed to FE and MF wastewater had 10-100 fold lower parasitemia than fish exposed to MCF or BS water (Fig. 7.3). Fish exposed to MF and FE wastewater continued to reduce their parasitemia to very low levels (<100 parasites/ml blood) for the remainder of the experimental period. In general, fish exposed to MCF wastewater or BS water had similar parasitemia levels for most of the observation period. However, during the mid-to-late stages of infection, goldfish in the MCF wastewater treatment had parasitemia 10 fold less than that of BS fish (Days 42-84). At the late stages of the infection (Days 70-84), all fish had a substantially reduced number of parasites in their blood and no differences were detected between wastewater treatment groups due to high variability between fish (Fig. 7.3)

Goldfish exposed to MF wastewater experienced the lowest survivorship of all treatment groups (50%); other treatment groups all experienced greater than 85% survivorship (Fig. 7.4). The survivorship of non-infected fish exposed to the municipal wastewater treatments and clean BS water was high: >77% survivorship (Fig. 7.5). Interestingly, a disproportionate number of MF fish died on Day 40 post-infection. Four of the fifteen remaining (27%) parasite infected-fish that had been exposed to MF wastewater and three of thirteen (23%) non-infected MF wastewater control fish died on this date. In the remaining non-infected fish, very low mortality occurred in fish exposed

to FE wastewater and no deaths occurred in those exposed to either the MCF wastewater or BS water treatments died over the entire experimental period (Fig 7.5). The reasons for the high mortality on Day 40 post-infection are not known, although it is likely associated with the membrane UF process rather than xenobiotics, as no deaths occurred in fish exposed to FE wastewater at this time point. Activated carbon treatment appeared to remove the substance that caused the mortality in fish exposed to MF wastewater, since no deaths occurred in goldfish exposed to MCF wastewater.

7.3 Discussion

The course of infection in goldfish exposed to different wastewaters was markedly different between the two individual experiments. The most conspicuous difference between the two experiments was the ability of the goldfish to clear an infection of the hemoflagellate protozoan parasite *T. danilewskyi* following xenobiotic exposure. In the Winter 2006 experiment fish exposed to MF wastewater, shown to contain higher levels of pharmaceutical residues and pesticides than that of MCF wastewater, were unable to successfully clear the parasitic infection and suffered high mortality, suggesting that a possible xenobiotic-induced immunosuppression occurred in these fish. However, during the Summer 2006 experiment, the course of infection in fish exposed to FE or MF wastewater given either the same parasite dose as the Winter 2006 experiment or a higher parasite dose had an enhanced clearance of *T. danilewskyi* trypomastigotes compared to their MCF or BS-exposed cohorts. While the fish exposed to MF wastewater in the Summer 2006 experiment also experienced high mortality, the majority of deaths occurred on Day 40 post-infection and were likely not associated with xenobiotic exposure.

In addition to the increased parasitemia observed in the Winter 2006 experiment goldfish exposed to MF wastewater, many of these fish developed lesions. These wounds often initially formed at the site of infection or tail bleeding site, but also formed *de novo* on the torso, mouth or fins of the fish. In fish exposed to MF wastewater, lesions were observed as early as Day 21 post-infection (three of eight fish) and progressed until 100% of infected fish had lesions (three fish; Day 63). Fish exposed to MCF wastewater in the winter of 2006 also formed lesions, but not until much later at Day 56 post-infection, and only 25% of surviving fish had lesions by the end of the experimental

period (two of eight fish; Day 84). Fish in the BS aquatics facility water had lesions form by Day 28 post-infection (one of ten fish), and by Day 84 five of eight fish had abnormalities such as inflammation of the tail or fin. All fishes' lesions worsened over time; no fish appeared to be able to heal from their infections. The increased prevalence and earlier onset of lesions in fish exposed to MF wastewater could be indicative of a possible xenobiotic-induced suppression of goldfish immunity during the Winter 2006 experiment, as supported by the increased parasitemia in this treatment group.

During the Summer 2006 experiment, however, goldfish exposed to FE and MF wastewater did not experience the increased parasitemia observed in the Winter 2006 experiment. Furthermore, these fish did not experience the high prevalence of lesions observed in the Winter 2006 experiment. In fish infected with the normal dose of 6.25 x 10^6 parasites, a small lesion formed on the tail of one of eight goldfish exposed to FE wastewater by Day 63 post-infection, but this wound did not worsen over time. Fish exposed to MF wastewater did not form any lesions throughout the experimental period, while only a small number of fish exposed to MCF wastewater showed signs of lesions or other abnormalities (two of eight fish; Day 63 post infection), one of which healed the lesion within a week. Clean water exposed fish (BS water) also experienced low rates of inflammation (one of ten fish; Day 28), and this fish was completely healed one week later.

Even infection of goldfish with a high dose of parasites did not induce a large number of lesions or physical abnormalities during the Summer 2006 experiment. Fish exposed to FE wastewater or BS water that were infected with a high parasite dose did not develop lesions at any time point. Some fish exposed to MF wastewater lost the ability to produce mucus and had raised scales, but these fish died shortly thereafter and this condition was observed during the time of typical peak parasitemia (Day 21; two of nine fish). One fish exposed to MCF wastewater that was infected with the high parasite dose experienced inflammation and hemorrhaging of the tail, but otherwise no lesions were observed (one of nine fish; Day 35 post-infection).

If xenobiotic exposure resulted in fish exposed to MF wastewater being in a potentially immunosuppressed state during the Winter 2006 parasite experiment and an immunologically primed state during the Summer 2006 experiment, the observed early

onset and high incidence of lesions only during the Winter 2006 experiment could support this hypothesis. However, it should be noted that following ~Day 21 post-exposure a substantial number of non-infected Winter 2006 fish exposed to FE, MF or MCF wastewater treatments developed lesions or fin degradations that would often not fully heal, whereas no lesions or morphological alterations were observed in any non-infected fish exposed to wastewater treatments during the Summer 2006 experiment. The possible cause(s) of the lesions are not known, as no epidemiological studies of the lesions were done.

A variety of factors may have affected the ability of fish exposed to the wastewater treatments to clear a parasite infection. It is possible that the large parasitemia differences observed between the Winter 2006 and Summer 2006 experiments may have been influenced by the mixture of xenobiotics present in the municipal wastewater at the time of exposure. Previous wastewater analysis shows substantial variability in the types and concentration of pesticides and pharmaceutical and personal care products in the FE and MF wastewater (see Chapter 4). However, this variation in xenobiotic concentration was only occasionally greater than 10-fold (2.4-D: 14-fold; MCPP: 39-fold; ibuprofen: 21-fold). It should be noted that since no gas chromatography/mass spectrometry analysis of wastewater samples took place in the Summer 2006 experiment due to budgetary reasons, it is unknown whether the fish were exposed to different concentrations or panels of xenobiotics between the Winter and Summer 2006 parasite studies.

The length of wastewater exposure prior to parasite infection differed slightly between trials; the Winter 2006 experiment fish were exposed to wastewater for approximately 21 days while the fish in the Summer 2006 experiment were infected following 7 days of exposure to wastewater. It was not expected that the difference in exposure time would influence the ability of goldfish to clear a parasite infection, as both 7 and 21 days are relatively short-term exposure periods. Interestingly, semi-quantitative RT-PCR analysis of goldfish immunological genes indicated a possible acute immunostimulatory effect at 7 days post-exposure in the kidney of fish exposed to FE and MF wastewater, specifically regarding tumor necrosis factor alpha (TNFα) and colony stimulating factor-1 receptor (CSF-1R) expression (see Chapter 6). It is possible

that the immune system of fish exposed to FE and MF wastewater during the Summer 2006 experiment may have been in a primed state upon infection with parasites after 7 days of exposure to wastewater. If so, then the immune system of these fish may have influenced the establishment phase of *T. danilewskyi*, resulting in the reduced mean parasitemia seen in Winter 2006 experiment FE and MF wastewater-exposed fish in the early-to-mid stages of infection. By Day 21 post-exposure, any possible xenobiotic-induced immunostimulatory effects (due to TNFα gene expression) were no longer evident.

A number of studies have demonstrated the modulating effects of pollutant exposure on fish immune response (4; 6; 7). By far, the majority of these studies have shown immunosuppressive effect of either individual xenobiotics (191; 193; 29; 30) or complex mixtures of chemicals (133; 122; 46) on fish immune responses. A compromised or improper immune response may manifest itself as a reduced ability to clear an infection with a pathogen. For example, Japanese medaka (*Oryzias latipes*) either injected with benzo[*a*]pyrene (BaP) or exposed to the pesticide malathion in the water were found to have significantly reduced resistance against *Yersinia ruckeri* bacterial infection than unexposed infected control fish (29; 30). In the case of BaP exposure, the reduced host resistance may have been due to BaP-induced suppression of lymphocyte proliferation, reduced numbers of antibody-forming cells and reduced free oxygen radical production (29; 30). Xenobiotic-induced immunosuppressive effects can result in an increased mortality rate following pathogen infection. Pascoe and Cram (191) observed that when cadmium-exposed three-spined sticklebacks (*Gasterosteus aculeatus*) were subsequently infected with pleurocercoids of the cestode *Schistocephalus solidus*, infected cadmium-exposed fish had significantly greater mortality than non-infected cadmium exposed fish.

Endocrine disrupting compounds may also induce immunosuppression of fish. Goldfish implanted with 50 mg/fish of the hormone 17β-estradiol (E2) were infected with 2.5×10^5 *T. danilewskyi* parasites pre fish were found to have significantly higher parasitemia and mortality than infected sham-implanted fish (193). It was hypothesized that the xenobiotic caused immunosuppression of the host, as E2 implanted fish were found to have significantly inhibited mitogen-stimulated leukocyte proliferation

compared to their unexposed cohorts (193). Interestingly, the authors of this study found that while E2 implantation reduced host resistance against the parasite, the ability of the fish to respond to subsequent challenge infection did not differ between exposed and control fish, indicating that the long-term protective immunity of the fish was not affected by E2 exposure.

Exposure of fish to complex mixtures of xenobiotics may also reduce host resistance to pathogens. When goldfish were exposed to a mixture of four common herbicides (atrazine, simazine, diuron, isoproturon; ~50 µg/L of each), Fatima *et al.* (46) observed immunological changes in herbicide-exposed fish that were correlated with an increased mortality following *Aeromonas hydrophila* bacterial challenge compared to unexposed fish. Reduced host resistance was also reported in fish isolated from PAH/PCB contaminated sites that were subsequently exposed to pathogens in a laboratory setting (122). A survey of native fish populations revealed that parasite prevalence and abundance were significantly greater in fish located close to polluted effluent discharge sites than those in fish from clean water reference sites (194).

Heterogeneous mixtures of man-made compound such as that found in sewage effluent have been shown to affect fish resistance against pathogens (133). Katuka (133) observed that goldfish exposed to various concentrations of either raw or treated municipal sewage effluent for thirty days experienced increased mortality upon infection with *Aeromonas salmonicida* than control fish. Specifically, the author found that exposure to as little as 5% treated effluent significantly induced higher mortality while exposure to greater than 20% raw effluent or 50% treated effluent resulted in 100% mortality of infected fish. We did not observe such high rates of mortality in FE wastewater exposed fish; however, parasite-infected fish exposed to MF wastewater had greater mortality compared to those in other treatment groups.

Some studies have demonstrated a reduction in parasitemia following pollutant exposure (195-197). For example, American plaice (*Hippoglossoides platessoides*) exposed to PAH and PCB contaminated sediments for five months were found to have reduced numbers of protozoan ectoparasites (*Trichodina spp.*) than control fish (197). Similarly, Siddall *et al.* (196) observed that naturally infected roach (*Rutilus rutilus*) exposed to 10% bleached kraft pulp and paper mill effluent for three weeks had

significantly fewer monogenean parasites of the *Dactylogyrus* genera on their gills than control fish. Pollutant exposure may also affect gastrointestinal parasites of fish. Khan and Kiceniuk (195) reported a reduction in the prevalence and intensity in both trematode parasite *Steringophorus furciger* infection of winter flounder (*Pleuronectes americanus*) as well as acanthocephalan *Echinorhychus gadi* infection of Atlantic cod (*Gadus morhua*) following exposure to crude oil contaminated water compared to clean-water exposed fish.

While we saw evidence suggesting that xenobiotic exposure may reduce the parasitemia (fish exposed to FE and MF wastewater during the Summer 2006 experiment), it should be noted, that the majority of the pathogens described in the above studies were ectoparasites or gastrointestinal parasites, and thus may have been exposed to higher concentrations of xenobiotics than a parasite living in the tissues of the host. It has been suggested that a reduction in parasitism in fish due to contaminants in the environment may be due to increased susceptibility of the parasite species to pollutants compared to that of the host species (198). It is equally possible that xenobiotics may alter the physiology of the host, which in turn could result in the loss of suitable parasite habitat (195).

Exposure of goldfish to xenobiotics in treated municipal wastewater or reuse water prior to infection with the hemoflagellate protozoan parasite *T. danilewskyi* yielded different results depending upon the experiment (Winter 2006 versus Summer 2006). Host-parasite interactions are complex, even in the absence of the inherent variability introduced into an experimental system utilizing real-time flow-through effluent exposure conditions. While it is difficult to precisely identify the factors that may have influenced our findings, it is clear that xenobiotic exposure in FE or MF wastewater influences the course of infection in *T. danilewskyi* infected goldfish compared to control conditions. Further study is necessary before the effects of differently filtered municipal wastewater exposure on the resistance of goldfish to parasitic infection may be fully elucidated.

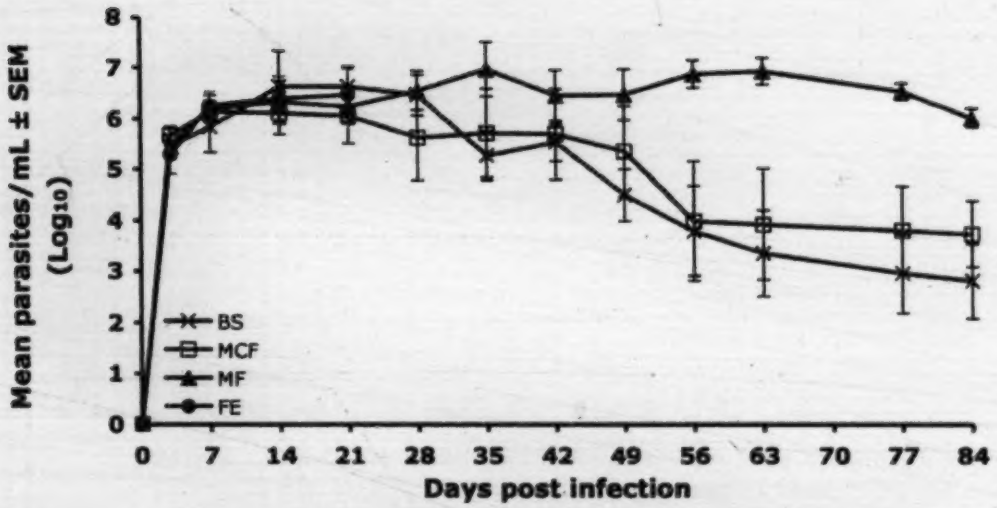

Figure 7.1 Mean parasitemia (± SEM) of Winter 2006 run goldfish exposed to different municipal wastewater effluents (final treated effluent, FE wastewater; UF membrane effluent, MF wastewater; and combined UF membrane and GAC unit effluent, MCF wastewater) that were subsequently infected with 6.25×10^6 parasites/fish. n = 10 fish for each wastewater treatment group at time of infection.

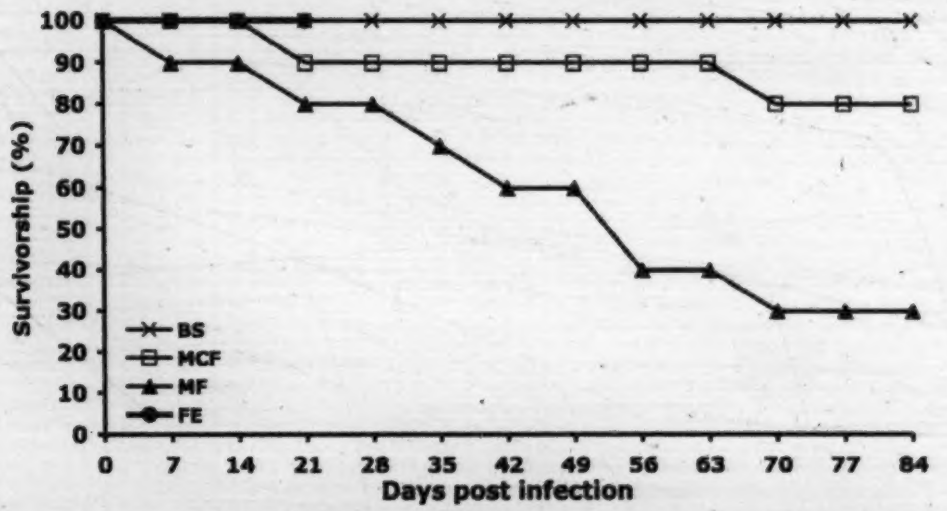

Figure 7.2 Survivorship of Winter 2006 run goldfish exposed to different municipal wastewater effluents (final treated effluent, FE wastewater; UF membrane effluent, MF wastewater; and combined UF membrane and GAC unit effluent, MCF wastewater) that were subsequently infected with 6.25×10^6 parasites/fish. n = 10 fish for each wastewater treatment group at time of infection.

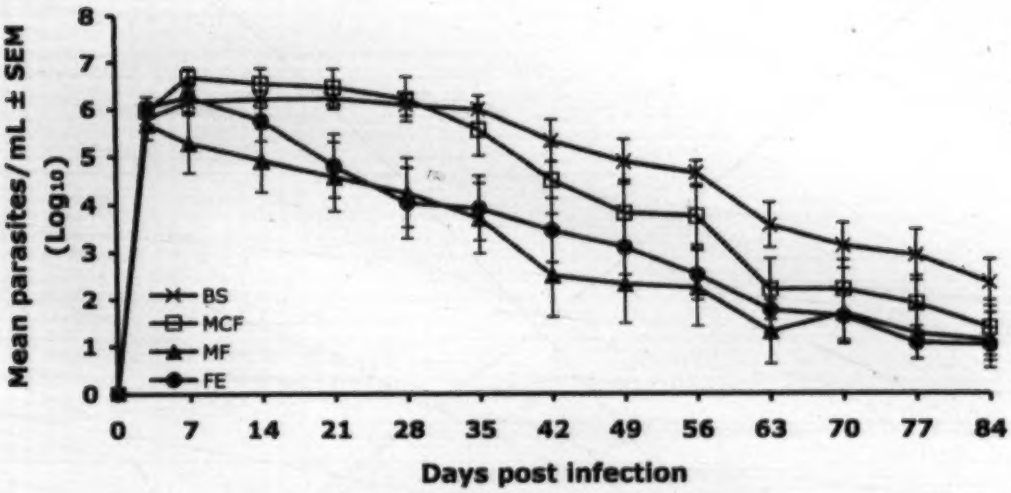

Figure 7.3 Mean parasitemia (± SEM) of Summer 2006 run goldfish exposed to different municipal wastewater effluents (final treated effluent, FE wastewater; UF membrane effluent, MF wastewater; and combined UF membrane and GAC unit effluent, MCF wastewater) that were subsequently infected with either 6.25×10^6 or 1.0×10^7 parasites/fish. n = 20 fish for each wastewater treatment group at time of infection.

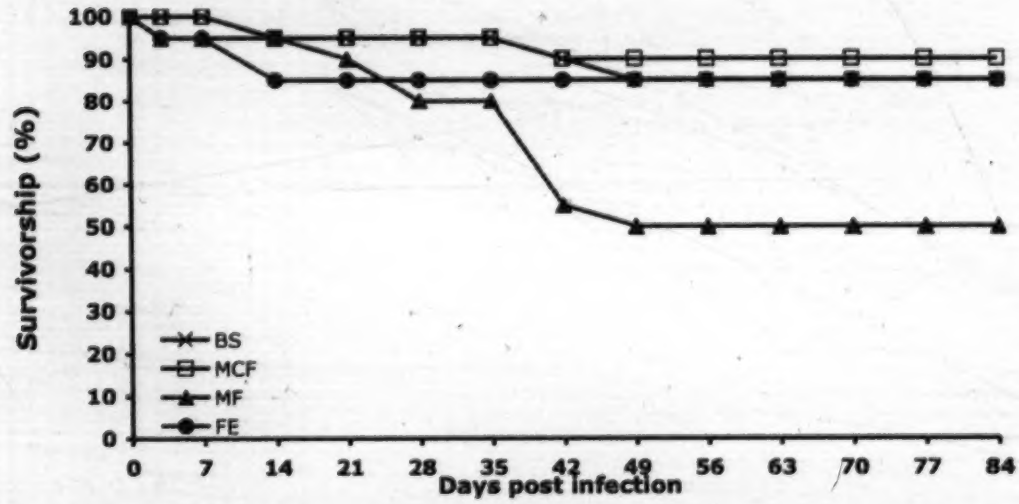

Figure 7.4 Survivorship of Summer 2006 run goldfish exposed to different municipal wastewater (final treated effluent, FE wastewater; UF membrane effluent, MF wastewater; and combined UF membrane and GAC unit effluent, MCF wastewater) that were subsequently infected with 6.25×10^6 or 1.0×10^7 parasites/fish. n = 20 fish for each wastewater treatment group at time of infection.

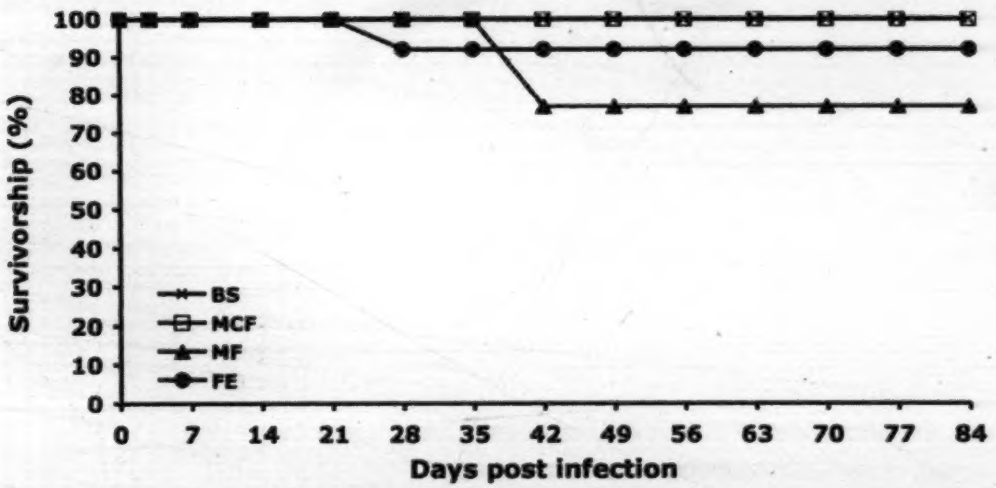

Figure 7.5 Survivorship of non-infected (control) Summer 2006 run goldfish exposed to different municipal wastewater effluents (final treated effluent, FE wastewater; UF membrane effluent, MF wastewater; and combined UF membrane and GAC unit effluent, MCF wastewater). n = 13 fish for both FE and MF wastewater fish, N = 12 for both MCF wastewater and BS water fish at time of infection.

CHAPTER EIGHT

GENERAL DISCUSSION

The main objective of my thesis was to examine the ability of goldfish to act as a biosentinel species for possible physiological effects caused by exposure to treated municipal sewage final effluent in a real-time flow-through system. Furthermore, I wished to examine if the length of exposure (7, 21, 60 or 90 days) impacted any potential toxicological effects in these organisms. My second objective was to examine the ability of re-use wastewater to reduce or eliminate concentrations of xenobiotics within the effluent compared to final effluent alone, and to reduce any associated physiological effects in exposed goldfish. This was accomplished by chemical analysis of wastewater treatment groups for the presence of a wide panel of man-made compounds and through the use of a number of different toxicological or immune bioassays in wastewater exposed goldfish.

The first part of my thesis research investigated the ability of reuse water methods, specifically membrane ultrafiltration with or without subsequent granular activated carbon (GAC) filtration (termed MCF wastewater and MF wastewater, respectively) to reduce or eliminate any xenobiotic contaminates that may have been present in treated municipal final effluent (FE wastewater). A number of xenobiotics were detected in the FE, MF and MCF wastewater samples, including pesticides, non-steroidal anti-inflammatory drugs (NSAIDs), anti-epileptic drugs, lipid lowering drugs and antibacterial compounds. These chemicals are commonly detected in municipal final effluent worldwide (1; 64; 42; 10; 2; 16; 65; 69; 49; 13; 50; 3; 60). A preliminary survey by the Government of Alberta in December of 2002 also examined xenobiotic contamination in the final effluent from Gold Bar Wastewater Treatment Facility (59). With the exception of naproxen, diclofenac, salicylic acid, triclosan and methyl triclosan, all the pharmaceuticals and personal care products (PPCPs) detected during my research were also found in this preliminary survey. The concentrations of xenobiotics detected during the Government of Alberta survey and that of my study varied considerably. However, this is not surprising considering the variability seen within even seven days of sampling in my study.

When the GAC unit was replaced on a frequent basis, MCF wastewater contained greatly reduced concentrations of both pesticides and PPCP compounds compared to FE wastewater. MF wastewater, however, did not consistently experience this substantial reduction of xenobiotics. Similar to our results, Clara *et al.* (65) found that ultrafiltration of sewage effluent through a membrane bioreactor did not lead to removal of select PPCPs or EDCs. Activated carbon adsorption, however, has been shown to be an efficient mechanism to reduce pollutants from contaminated solutions (152; 141). In our experiment, the removal efficiency in MCF wastewater, and to a lesser extent, in MF wastewater, varied depending upon the chemical compound, its original concentration in the FE wastewater, and the sampling date. For example, while the NSAID ibuprofen concentration in MF wastewater did not differ from that of FE wastewater in the Spring/Summer 2005 experiment, high ibuprofen removal efficiency occurred in both MF and MCF wastewater in the Winter 2006 experiment.

Ultrafiltration prior to activated carbon filtration was advantageous as it resulted in the removal of bacterial and all other particulate matter greater than 0.04 μm in diameter, possibly extending the life of the carbon filter. However effective as activated carbon filtration was at removing PPCPs and pesticides from municipal wastewater, it is unlikely that this method will ever be used on a large scale in a wastewater treatment plant such as Gold Bar Wastewater Treatment Plant. This is because of the large amount of activated carbon required and the associated labour necessary to treat the large volume of wastewater effluent produced by the facility on a daily basis. More efficient large-scale xenobiotic removal from municipal sewage effluent would likely occur through enhanced biological or ozone treatment, for example. For the purpose of this experiment, however, granular activated carbon filtration successfully removed PPCP compounds and other bioactive molecules from the wastewater so long as the GAC unit was replaced every three to four weeks and breakthrough of xenobiotics did not occur.

Chemical analysis of xenobiotics in municipal wastewater is limited in that it does not predict what, if any, effects these chemicals may have on living organisms. While single cell-based tests such as the estrogen screen (E-screen) or yeast estrogen screen (YES) assay are quick and cost-effective methods to screen large numbers of samples for xenobiotic contamination, these assays may not necessarily reflect the full impact of

these compounds on an animal's fitness or physiology. Interactions between different xenobiotics may result in supra-additive effects on an organism that cannot always be predicted using results from individual exposure studies (199). Though studies have suggested that no appreciable human health risk exists from the presence of PPCP compounds in surface and drinking water at this time (200), these compounds can affect the physiology of aquatic organisms (4-8), even at low concentrations (44; 111; 45-47). In order to determine the true physiological consequences of wastewater exposure it is necessary to use a whole animal model such as teleost fish. Chapters 5-7 of my thesis research examined the potential toxic effects of FE wastewater compared to that of MF or MCF wastewater on goldfish (*Carassius auratus*) physiology.

I chose to use an aquatic vertebrate, the goldfish, as a biosentinel organism for possible physiological effects of sewage wastewater or reuse wastewater exposure. Goldfish were chosen over other teleost species such as fathead minnows (*Pimephales promelas*), zebrafish (*Danio rerio*), or rainbow trout (*Oncorhynchus mykiss*) for a number of reasons. Goldfish are more resistant than rainbow trout to the fluctuations in dissolved oxygen that could occur in wastewater effluent, and thus more likely to survive a long-term exposure study in which various basic water quality parameters may vary considerably. Goldfish are also of a much larger size than fathead minnow or zebrafish. Because of this, a larger quantity of tissue can be obtained from each fish, potentially allowing each exposed fish to be efficiently used for a number of different assays. Furthermore, as aquatic organisms they are constantly exposed to pollutants in their environment and may exhibit toxic effects due to wastewater exposure prior to that of an organism that encounters contaminants on an occasional basis. However, while fish can serve as biosentinels for pollutant exposure and as powerful tools in pollutant exposure risk assessment, it is essential to be aware of the vastly different physiological responses that can occur depending upon the xenobiotic species examined, exposure concentration, duration of exposure, as well as the species and life stage of the organism exposed to the contaminant (18).

The 7-ethoxyresorufin-O-deethylase (EROD) assay is a common biomarker of aryl hydrocarbon exposure in organisms. This assay quantitatively measures the catalytic activity of the cytochrome P450 (CYP1A) enzyme family to convert 7-ethoxyresorufin

into its fluorescent product, resorufin. CYP1A proteins are involved in the biotransformation of aromatic hydrocarbons such as PCBs, PAHs and pesticides into more excretable forms (5), and numerous studies have shown the upregulation of EROD enzymatic activity following exposure to a variety of man-made compounds (22; 201; 121). My research has presented evidence that both FE and MF wastewater are weak EROD inducing mixtures, implying that the membrane UF does not substantially remove aryl hydrocarbon residues from FE wastewater. This induction of the P450 (CYP1A) enzyme activity was more evident in the Winter 2006 experiment than the Spring/Summer 2005 experiment. Furthermore, the importance of proper replacement of the GAC unit was illustrated by the significant induction of EROD activity in MCF wastewater exposed fish at Day 60 during the Winter 2006 experiment. Throughout both the Spring/Summer 2005 and Winter 2006 experiments, no cumulative effects of chronic wastewater exposure were observed within goldfish exposed to either FE or MF wastewater. This was supported by the observation that the substantial increase in EROD activity seen in fish exposed to MCF wastewater at Day 60 was no longer evident by the Day 90 sampling time point. Other studies have also found that exposure of teleost fish to municipal FE wastewater induces a similar fold increase in EROD activity compared to control fish (128; 32). To the best of my knowledge, my study is the first to show that ultrafiltration of this final effluent wastewater does not reduce the level of P450 (CYP1A) enzymatic activity in exposed goldfish liver microsomes, and thus likely is inefficient at removal of aryl hydrocarbon receptor agonists from sewage effluent.

Examination of male goldfish plasma collected at the various sampling time points throughout the experiment also revealed a significant feminization effect in both FE and MF wastewater exposed fish. Vitellogenin (Vtg) is a female-specific egg yolk precursor protein that is naturally found in the plasma of sexually mature female fish. However, the gene encoding for Vtg is not sex linked and thus the Vtg protein may be induced in sexually immature fish or male fish following exposure to natural estrogens (112) or other xenoestrogens found in municipal sewage wastewater (23; 25; 129; 32; 131; 130). My research has confirmed that exposure of male teleost fish to municipal sewage treatment plant FE wastewater results in a significant induction of plasma Vtg, and has shown that membrane UF wastewater (MF) can also cause feminization in male

goldfish. While Li *et al.* (202) found a significant induction of Vtg (ranging from 0-398 μg/mL) in wild *C. auratus* caught in a river downstream of sewage treatment plant effluent discharge sites, others (203) did not detect any Vtg feminization effect in goldfish following exposure to river water downstream of sewage treatment plants compared versus reference site fish. The concentrations of xenobiotics, such as estrogens, in final sewage effluent is highly variable however, and may be influenced by such factors as the influent concentration, the type of sewage treatment used (140) and the temperature at which treatment occurs (13).

No Vtg synthesis was induced in male fish exposed to MCF wastewater, indicating that activated carbon filtration is an effective method to remove estrogenic EDCs. Activated carbon filtration has been previously shown to remove estrogenic compounds from polluted wastewater. Similar to our results, Coors *et al.* (147) found that aerobic biological degradation of municipal landfill leachate followed by ultrafiltration and adsorption to activated carbon filtration completely removed any estrogenicity activity from the leachate, as determined using reporter gene expression in a human breast cancer derived cell line (MVLN cells).

Though we did not measure the concentrations or relative estradiol equivalents of specific EDCs in our different wastewater treatments, previous studies have estimated that final effluent from Gold Bar Wastewater Treatment Plant can have ~20 ng/L of total estrogenic equivalent activity (75). Furthermore, sampling done by the Alberta Government also at Gold Bar detected concentrations of 2.08 ng/L 17β-estradiol (E2), 34.06 ng/L estrone, and 2391.92 ng/L of nonylphenol in a grab sample of final effluent during December of 2002 (59).

Different EDCs have variable potencies in terms of eliciting a physiological response in exposed organisms due to heterogeneous binding affinity to the animal's estrogen receptor. However, it is commonly assumed that if an organism is exposed to mixtures containing several estrogenic EDCs, the xenoestrogens should act in an additive fashion on the physiology of the exposed organism. Interestingly, recent research has questioned this belief. Lin and Janz (45) exposed zebrafish (*Danio rerio*) to binary mixtures of the weak estrogen receptor agonist nonylphenol and the potent estrogen receptor agonist EE2 and found that these mixtures may act in an additive or non-additive

manner depending upon the relative concentrations of each EDC. Vitellogenin was induced in an additive fashion upon exposure to 1 ng/L 17α-ethinylestradiol (EE2) and 10 μg/L nonylphenol, but higher concentrations of nonylphenol in conjunction with 1 ng/L EE2 resulted in a lower level of Vtg induction in fish compared to fish exposed to 1 ng/L EE2 alone. The authors suggest that this antagonism may be due to competition between the two EDCs at the estrogen receptor site (45). Consequently, care must be taken when the total estradiol equivalent activity (EEQ) of mixtures such as sewage effluent is predicted through the addition of EEQ's from each HPLC fractionation, as the calculated estrogenicity may not necessarily correlate with the true estrogenic effects in exposed organisms.

Binary mixtures of xenobiotics may not merely impact a bioassay such as Vtg induction alone. Kirby *et al.* (204) exposed flounder (*Platichthys flesus*) to the waterborne EROD inducer dibenz[a,h]anthracene (DbA) and various concentrations of the estrogenic compounds E2, EE2 or nonylphenol for 10 days before measuring both EROD activity and Vtg induction in these fish. The authors found that the concentrations of EE2 and nonylphenol that caused significant Vtg induction were paralleled by a corresponding inhibition of EROD activity. In fish exposed to DbA and various concentrations of E2, EROD activity was significantly reduced at E2 levels approximately 100-fold lower than that required to induce a significant induction of Vtg protein (204). This suggests that in complex mixtures in which both aryl hydrocarbon receptor agonists and xenoestrogens are present, such as municipal sewage effluent, the presence of estrogenic compounds may suppress EROD activity more than would be otherwise observed, an observation that may have impacted the measured EROD activity in my study.

EROD activity can also be reduced following exposure to pharmaceuticals found in municipal wastewater. Carp (*Cyprinus carpio)* hepatocytes incubated *in vitro* with various xenobiotic compounds such as gemfibrozil, diclofenac and the anti-depression drugs fluoxetine, fluvoxamine and paroxetine were found to have inhibited activity of CYP1A enzyme and other enzymatic isoforms (109). When diclofenac was administered *in vitro* with an inducer to rainbow trout (*O. mykiss)* hepatocytes, EROD activity was reduced compared to that of cells that were not exposed to the NSAID (108).

Since the FE and MF wastewater exposure conditions in this study contained both EROD inducing chemicals, estrogenic compounds and PPCPs, the potential intricate interactions of these compounds may have resulted in much more complex physiological reactions than if goldfish were exposed to these xenobiotics individually. Mixture toxicity studies such as the ones discussed above question the applicability of individual-xenobiotic exposure studies to that of real world environments, where complex mixtures of chemicals are the norm.

Xenobiotic exposure commonly results in immunosuppressive effects in teleosts (6; 7; 17). Pollutants have been shown to impact such aspects of the immune response as mucus production on the gill (126), the relative proportions of leukocytes found in tissues (133; 30; 135), or the relative activity of immune cells, as measured by such factors as their ability to undergo proliferation (132; 128), phagocytosis (181), or to produce such substances as oxygen radicals (27; 30; 181; 183), cytokines (31) or antibodies (181; 123; 31). I chose to examine the immunological response of goldfish to wastewater treatments through mitogen-stimulated peripheral blood leukocyte (PBL) proliferation and the expression of immunologically relevant genes through semi-quantitative RT-PCR.

Overall, wastewater exposure did not induce any non-specific changes in un-stimulated PBL proliferation, and consequently, for subsequent analysis a ratio of the absorbance of mitogen-stimulated cells over that of un-stimulated absorbance values was determined for each treatment group and time point. Exposure to wastewater that contained higher levels of xenobiotics (FE and MF wastewater) did not consistently impact the mean proliferation ratio. However, fish exposed to these wastewater conditions at Day 21 post-exposure had a higher proliferative ability than that of MCF wastewater exposed fish. This suggests that xenobiotic exposure may have resulted in these fish having an acute immunostimulatory ability that manifested itself as an increased ability to proliferate their PBLs upon exposure to a mitogen at Day 21 post exposure. If this increased proliferative ability of PBLs corresponded with an increased number of circulating leukocytes, fish exposed to FE and MF wastewater may have had an enhanced ability to fight off an infection. Other studies have also shown an augmented PBL mitogen-stimulated proliferation following exposure to treated sewage effluent in fish (135).

The later time points of FE and MF wastewater exposure in my study did not parallel this increase in PBL proliferation; in fact, an overall immunosuppression of the PBL proliferation ratio was observed at Day 60 and Day 90 exposure time points regardless of wastewater exposure group. Reasons for this uniform reduction in PBL proliferation are not known. However, Kątuka (133) observed that exposure of goldfish to diluted sewage effluent caused a reduction in the numbers of circulating leukocytes at later sampling time points. This could have been due to a reduced proliferative ability of these cells, similar to the results observed in my study.

Gene expression studies also suggest that xenobiotic exposure may cause a short-term immunstimulatory or priming effect on goldfish physiology, as the relative expression of the proinflammatory cytokine, tumor-necrosis factor alpha (TNF-α), and the receptor for the monocytes and macrophage growth factor, colony stimulating factor-1 (CSF-1R), were both increased early in the exposure study. However, other immunologically relevant genes, such as the evolutionarily conserved pathogen associated molecular pattern receptor, toll-like receptor-22 (TLR-22), or another immune cell growth factor, granulin, were either unaffected by wastewater exposure (TLR-22) or ubiquitously down-regulated (granulin) at early time points. Furthermore, in all genes examined, expression levels did not differ from that at control fish at the later time points; time points during which PBL proliferation ratios were uniformly reduced compared to control fish.

While Moens *et al.* (166) did not detect any changes in immune gene expression levels following exposure of juvenile carp to industrial effluent, others (31) have found immunosuppressive effects of pentachlorophenol (PCP) on crucian carp leukocytes TNF-α and interleukin-1 beta (IL-1β) cytokine mRNA expression when cells were exposed *in vitro*. Marchand *et al.* (205) also observed changes in the expression of genes related to the immune system (IgM heavy chain constant region and peptidoglycan recognition protein) in European flounder (*Platichthys flesus*) following pesticide exposure, as well as changes in genes related to metabolism, signal proteins, and cytoskeletal transport proteins. A reduction in gene expression may be correlated with a reduction in the activity of the animal's immune cells. For example, in a previous study by Chen *et al.* (31), the authors found that *in vitro* crucian carp head kidney leukocyte PCP exposure

resulted in a significant reduction in reactive oxygen intermediate production and phagocytic ability of these cells, as well as a reduced IgM titre in the serum of fish exposed to PCP concentrations as low as 0.053 mg/L *in vivo*. Interestingly, some studies have found that exposure of rainbow trout head kidney leukocytes to PCB126 causes a transient (2-4 hrs post exposure) stimulation of IL-1β gene expression rather than long-term damage to the immune system (167).

The majority of toxicological studies have observed immunosuppression in xenobiotic-exposed fish, either through exposure to individual xenobiotics in a laboratory setting or to complex mixtures of chemicals in polluted environments (27; 133; 122; 4; 29; 6; 7; 30; 17; 44; 181; 31; 180; 46). Since immunosuppression of an organism may result in an increased number of pathogens upon infection, we examined whether goldfish exposed to the different wastewater treatments experienced changes in parasitemia upon challenge with the parasite *Trypanosoma danilewskyi*.

During the Winter 2006 experiment, parasite-infected fish exposed to MF wastewater were unable to clear the infection and suffered a high degree of mortality. Furthermore, these fish suffered an increased prevalence and earlier onset of fin and body lesions than those exposed to MCF wastewater or clean water (BS). Alternatively, both parasite-infected fish exposed to MCF wastewater and BS water had similar parasitemia to each other throughout the experiment, both had a high degree of survivorship, and both were able to successfully eliminate the parasites over the infection period. Taken together, the data suggests that fish exposed to MF wastewater experienced an immunosuppression due to xenobiotics present within the MF effluent, and this immunosuppression led to increased parasitemia at later stages of infection, an increased mortality, and a higher prevalence of lesions compared to fish not exposed to these higher concentrations of xenobiotics.

Both an impaired ability to clear a pathogen infection and a reduced survivorship following xenobiotic exposure has been previously observed in fish (133; 122; 28-30). For example, when Chinook salmon (*O. tshawytscha*) were collected from either a clean water reference site or a site contaminated with PAH/PCB mixtures, fish from the polluted site had significantly reduced resistance against the bacteria *Vibrio anguillarum* in a subsequent laboratory challenge (122). These immunological changes may also be

elicited by sewage wastewater exposure. Infection of male rainbow trout with inactivated *Aeromonas salmonicidia* bacteria after exposure to diluted treated sewage effluent resulted in no enhancement of serum lysozyme activity, while clean water cohort fish did experience a significant serum lysozyme activity enhancement six weeks later (136).

During the Summer 2006 repeat of the previous parasite infection experiment, rather than seeing the same trend of immunosuppression in xenobiotic-exposed fish infected with *T. danilewskyi* parasites, the mean parasitemia in fish exposed to FE and MF wastewater treatments was lower than that of MCF wastewater or BS water fish. This reduction in parasitemia in fish exposed to FE and MF wastewater during the mid-stages of infection indicates a possible immunostimulatory effect of xenobiotic exposure in exposed goldfish. Furthermore, compared to the Winter 2006 experiment, very few lesions were observed in infected fish exposed to municipal wastewater or BS water treatments, supporting the hypothesis that FE or MF wastewater exposure in parasite infected goldfish did not result in immunosuppression during the Summer 2006 experiment.

Possible reasons for the discrepancy between parasitemia in the Winter 2006 and Summer 2006 experiment fish include natural or seasonal changes in the composition and concentration of xenobiotics in the wastewater, and the number of days fish were exposed to the wastewater treatments prior to infection. Fish infected with parasites during the Summer 2006 experiment were exposed to wastewater treatments for only 7 days prior to challenge, whereas the Winter 2006 experiment fish were exposed to wastewater treatments for ~21 days prior to infection. At Day 7 post exposure, the expression of TNFα and CSF-1R were both significantly increased in fish exposed to FE and MF wastewater. If the increase in TNFα and CSF-1R gene expression corresponded with an increased proinflammatory response, and enhanced proliferation and survival of monocytes and macrophages in these fish, respectively, fish infected at Day 7 post-exposure (Summer 2006 experiment fish) may have been in an immunologically primed state compared to their MCF and BS exposed cohorts. As such, the observed reduction in parasite load in these fish may have been due to this immunostimulation, resulting in a better ability to control the *T. danilewskyi* infection. By Day 21, the time point of the initial Winter 2006 infection study, the induction of TNF-α was no longer evident. This

could have eliminated any xenobiotic induced-immunostimulatory effects, resulting in the increased parasitemia values observed within the fish exposed to MF wastewater during the Winter 2006 experiment.

It is unlikely that infection of goldfish with *T. danilewskyi* would be a viable method to assay for possible toxic effects of municipal wastewater or reuse water. This parasite-host model system, in addition to the variable effect on immunocompetence observed between the Winter 2006 and Summer 2006 experiments, was very labour intensive. Any effects of wastewater exposure on mean parasitemia within infected fish was generally not observed until several weeks following challenge – far too late if the fish are to be used to detect possible toxic effects of xenobiotic exposure in the receiving environment or for society.

Based upon data collected using semi-quantitative RT-PCR of key immunological genes, mitogen-stimulated PBL proliferation at Day 21 post exposure, and the Summer 2006 experiment parasite study, exposure to FE and MF municipal wastewater may result in a slight stimulatory effect on the immune system of the goldfish. However, care must be taken when interpreting these results due to the complexity of the vertebrate immune response and the fact that only a select number of immunological parameters were examined in this study. The proliferation of PBLs and the relative expression level of TLR-22, TNF-α, CSF-1R, and granulin mRNA are only a fraction of the many immunological genes or assays that can be used to examine the effect of xenobiotic exposure in fish. Furthermore, it is not known whether any observed changes in gene expression resulted in alterations in the function or protein expression of these encoded molecules, nor if an increased proliferative ability of the magnitude observed on Day 21 post-exposure would yield any benefits in pathogen defense.

Interestingly, one of the major assumptions regarding the use of biomarkers in toxicological studies is that the biomarkers should respond in a dose-dependant manner to increasing or decreasing xenobiotic concentrations. However, the theory of hormesis – that the response of an organism to a compound may result in stimulatory effects at low concentrations and inhibitory results at high concentrations of the effecter substance, has been shown to be a relatively common and reproducible event in biology (206). Many toxicological studies show adverse effects of chemical exposure, but only at xenobiotic

concentrations many folds higher than that found in the environment or even undiluted sewage effluent. Consequently, it is possible that the lower concentrations and varied mix of xenobiotics detected in FE and MF wastewater induced the acute induction of the immunological parameters observed in this study through hormesis.

Future Research

Future research could involve the use of additional bioassays in examining the possible toxic effects of exposing goldfish to treated municipal wastewater or reuse water. The induction, suppression or even consistent expression of genes compared to control fish may help shed light onto the effects of xenobiotic mixtures on an animal's physiology. For example, the relative expression level of additional immunologically relevant genes could be examined in the head kidney or other tissues. These genes could include inducible nitric oxide synthase (iNOS), the enzyme responsible for the production of nitric oxide - a reactive nitrogen metabolite involved in innate immunity and antimicrobial activity. Several other cytokines could also be examined, including the pro-inflammatory cytokine IL-1, or IL-10, a cytokine involved in the formation of a T_H2 humoral response. My laboratory also has primers available for transforming growth factor beta (TGFβ), a cytokine involved in controlling an immune response through inhibiting the growth of certain cell types. Furthermore, the expression of the ligand for CSF-1R, M-CSF, could be analysed to help determine whether the induction of CSF-1R could have been due to an increase in M-CSF expression.

Alternatively, a DNA microarray, as developed for the closely related common carp [*Cypinus carpio* (166)], could be used to simultaneously screen the relative expression levels of a large number of genes in wastewater-exposed goldfish compared to unexposed fish. This could allow us to have an increased understanding of the interactions of hundreds of genes simultaneously compared to examining individual genes alone. If interesting trends were then observed, the expression of individual genes could be more closely examined using tradition semi-quantitative RT-PCR techniques or real-time PCR.

One of the limitations of using PCR techniques is that the actual protein expression or biological activity of the target genes is often not known. However, my laboratory has available the proper equipment and techniques to determine the prevalence

of CSF-1R protein on the surface of fish immune cells from fish exposed to wastewater using fluorescently labeled anti-CSF-1R antibodies and flow cytometry. This technique would allow us to examine if wastewater treatment or the length of exposure impact the relative proportion of monocytic lineage cells in the leukocyte population compared to control fish, as the expression of this receptor has been shown to increase as cells progress from monocytes to macrophages (177).

Studies have demonstrated the inhibitory effects of xenobiotic exposure on a variety of immunological parameters that may also be potentially studied in goldfish exposed to municipal wastewater (4; 6). These parameters include serum lysozyme activity, which measures the activity of antimicrobial enzymes (132; 136), and the production of reactive oxygen intermediates (192; 183; 207). Reactive oxygen intermediates can indicate the relative activity of immune cells and, when produced in excess, result in increased peroxidative damage in tissues. Other biomarkers may also be used to determine effluent-induced physiological changes in goldfish. Fish can become immunosuppressed when exposed to environmental stressors. In periods of stress, the metabolic activity of fish is elevated during which glucose is one of the primary sources of fuel (208). Consequently, the relative plasma glucose or liver glycogen levels may shed insight into the effects of wastewater exposure on chronic stress within these fish.

In addition to measuring the concentration of inducible reproductive proteins such as Vtg, physiological effects of EDCs within municipal wastewater may be measured in a more subtle and sensitive manner by radioimmunoassay (RIA) of reproductive hormones or cholesterol. For example, RIA has been used to successfully detect changes in plasma testosterone in goldfish following exposure to the phytoestrogen beta-sitosterol (209).

Another common bioassay that could be used in future studies is the accumulation of xenobiotics within wastewater-exposed fish. The NSAID diclofenac was found to bioaccumulate in a dose-dependant manner in the liver of rainbow trout exposed to this pharmaceutical for 28 days (19). This bioaccumulation has also been observed in other fish species exposed to different pharmaceutical compounds. Goldfish exposed to the lipid regulating drug gemfibrozil at environmentally relevant concentrations had bioconcentration factors of 113 times that found in the surrounding water, and this bioaccumulation was linked to parallel physiological changes in the fish (116). Due to

the presence of PPCP compounds in the FE and MF wastewater samples in this study, gas chromatography/ mass spectrometry of various tissue from goldfish exposed to municipal wastewater could allow us to more closely predict what, if any bioaccumulation occurs in chronically exposed organisms in the environment and if accumulation of PPCPs or other xenobiotics are linked to physiological effects within these fish. Furthermore, the presence of xenobiotics in the tissues of fish may lend insight to chemicals that the fish was previously exposed to – compounds that may have not been detected in periodic chemical analysis of wastewater samples.

Another method used to remove xenobiotics from sewage wastewater, ozonation, has been show to reduce PPCPs and EDCs concentrations by as much as >90% over the influent values (210). In addition to reducing the concentration of these compounds, the biological effects of wastewater exposure may also be reduced by ozonation. Estrogenic activity in primary or secondary municipal effluent has been shown to be reduced to extremely low levels following ozonation, as measured using *in vitro* methodology (211; 212). Reductions in the toxic effects elicited by municipal wastewater exposure may also be observed using animal models *in vivo*. Gagné *et al.* (213) exposed of freshwater mussels (*Elliptio complanata*) using a real-time flow system to different dilutions of primary treated sewage effluent or to ozonated effluent. Ozonation either reduced the intensity of the toxic response or resulted in an increase in the concentration of effluent required to elicit a statistically significant response in the mussels. However, the authors did find that lipid peroxidation was increased in the gills of mussels exposed to ozonated effluent, suggesting that ozonation may increase oxidative stress within these animals. Future research could compare the ability of ozonation versus activated carbon adsorption in reducing chemical contaminants from municipal wastewater, and these technologies' effects on physiological changes in exposed goldfish.

The use of real-time flow-through exposure conditions allows us to determine the overall net effects of municipal final effluent and reuse water on goldfish physiology. The ability of goldfish to act as biosentinel organisms for xenobiotics in municipal wastewater, as shown through my studies, will greatly enhance our ability to assess the efficacy of new chemical removal technologies on a variety of whole animal physiological parameters – ranging from xenobiotic removal to endocrine disrupting

compounds or even an animal's immune response, with the potential for many other toxicological assays to be tested within this system. Consequently, through studies such as this, we may be able to determine the most efficient way to ensure that treated municipal wastewater or reuse water has the minimum impact upon exposed organisms and future generations.

CHAPTER NINE

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